The Synaptic Cell Adhesion Molecules SynCAMs Are Involved in Early Sensory Axon Pathfinding

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1. Summary

Neural circuits are the basic units of a functional nervous system. For their formation multiple, well-orchestrated processes are required, including neuronal migration, axon elongation, axon guidance, synapse formation and maintenance, as well as synaptic plasticity. The synaptic cell adhesion molecules SynCAMs are members of the Ig-superfamily of cell adhesion molecules (IgSF-CAMs). Originally, they have been identified at the synapse, promoting synaptogenesis and regulating synaptic plasticity. Additionally they have been implicated in myelination of axons in the central as well as the peripheral nervous system. In addition to the function of SynCAMs in these late stages of neural circuit formation, they were recently implicated in earlier steps of development, namely in the guidance of post-crossing commissural axons at the midline of the chicken spinal cord.

In the present study, we could show that the function of SynCAMs in early axon guidance depends on their complex interaction pattern, including homo- and heterophilic cis- as well as homo- and heterophilic trans-interactions. We suggest that the specific composition of the SynCAM complexes elicits different intracellular responses in axons and growth cones. Indeed, changing the expression levels of SynCAMs resulted in a disorganization of sensory axonal networks due to altered axon-axon contacts. In agreement with a function of SynCAMs in regulating selective fasciculation between sensory axons, in vivo perturbation of these molecules by in ovo RNAi resulted in aberrant pathfinding of sensory afferents in the dorsal spinal cord of the chicken embryo. Taken together, we provide strong evidence that SynCAMs are required for early neural circuit formation. Thus, SynCAMs not only contribute to the establishment of a functional nervous system by mediating synaptogenesis and myelination but also by ensuring correct axon guidance.

2. Zusammenfassung

Neuronale Netzwerke sind die Grundbausteine eines funktionierenden Nervensystems. Deren Entstehung ist abhängig von vielen, koordinierten Prozessen, wie zum Beispiel der Migration von Neuronen, dem Wachstum und der Navigation von Axonen aber auch der Bildung, Erhaltung und Plastizität der Synapsen. Die SynCAMs sind eine Gruppe von Proteinen, die zur Superfamilie der Immunoglobulin-ähnlichen Zelladhäsionsmoleküle gehört. SynCAMs wurden erstmals als synapseninduzierende Moleküle identifiziert. Zudem wurde ihnen eine Funktion in der Regulation der synaptischen Plastizität und in der Myelinisierung von zentralen und peripheren Axonen zugeschrieben. Dies sind Prozesse, die spät in der Entwicklung des Nervensystems stattfinden. Im Gegensatz zu diesen späten Funktionen wurde kürzlich gezeigt, dass SynCAMs schon viel früher als Wegweisermoleküle für die Navigation von post-kommissuralen Axonen entlang der Längsachse des sich entwickelnden Rückenmarks benötigt werden.

In der vorliegenden Studie konnten wir zeigen, dass die axonale Navigation vom komplexen Interaktionsmuster der SynCAMs abhängig ist. SynCAMs können sowohl homo- als auch heterophil in cis- und in trans-Orientierung miteinander interagieren. Die spezifische Zusammensetzung der SynCAM-Komplexe könnte kontrollieren, welche intrazellulären Signale in Axonen und deren Wachstumskegeln ausgelöst werden und so deren Verhalten bestimmen. Tatsächlich fanden wir ein verändertes Verhalten in der Ausbildung von Kontakten zwischen sensorischen Axonen, welches aghängig von der Expression der SynCAMs war. Übereinstimmend mit den Daten der funktionellen in vivo Analyse deutete dies darauf hin, dass SynCAMs die selektive Faszikulierung zwischen sensorischen Axonen regulieren. Sensorische Axone waren unfähig ihren Weg im dorsalen Rückenmark des Hühnerembryos zu finden, wenn die Expression der SynCAM Moleküle experimentell verändert wurde. Die Daten dieser Studie beweisen, dass SynCAMs bereits in frühen Prozessen der Netzwerkentwicklung eine wichtige Rolle spielen. Demzufolge beteiligen sich SynCAMs nicht nur an Synapsenbildung und Myelinisierung, sondern gewährleisten auch, dass Axone ihr richtiges Zielgewebe finden. So tragen diese Moleküle auf verschiedenen Entwicklungsebenen zur Entstehung des Nervensystems bei.

3. Introduction

Sensory neural circuit formation in the chicken embryo

Formation of dorsal root ganglia (DRGs)

After neurulation, NCCs start to emigrate from the neural tube at Hamburger and Hamilton stage (HH) 11 of the chicken embryo (Bronner-Fraser & Fraser, 1989; Krispin *et al.*, 2010) (Fig. 1A-C). They migrate along stereotypic pathways to give rise to a variety of neuronal and non-neuronal cells. Cells delaminating form the neural tube along the ventral pathway develop into cells of the dorsal root ganglia (DRG) and cells of the sympathetic ganglia (SG) whereas cells migrating along the dorsolateral pathway give rise to melanocytes (Fig. 1D).

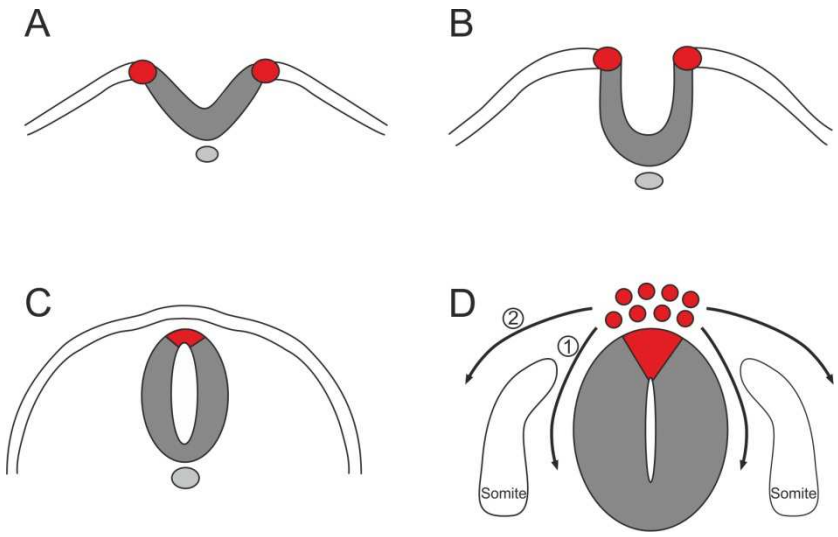

Figure 1. Neurulation and neural crest cell migration. (A-C) During neurulation the neural plate (A, grey) folds up right above the notochord (light grey) to form the neural groove (B) and finally closes to generate the neural tube (C). At the hinge point between neuroectoderm (grey) and ectoderm (white) the population of neural crest cell precursors arises (red). (D) NCCs emigrate from the dorsal neural tube (red) and migrate along different pathways, either ventrally between neural tube and somites (1) or dorsolaterally between somites and epidermis (2). NCCs delaminating along the ventral pathway (1) give rise to DRGs and sympathetic ganglia. Migration along the dorsolateral pathway (2) generates melanocytes.

It is under debate whether NCCs are a homogeneous multipotent population of precursors adopting their fate during migration in an environment-dependent manner or whether they are fate restricted before emigration from the neural tube. The first model shows that premigratory and emigrating NCCs remain multipotent and that fate restriction occurs during or after migration according to the environment they encounter (Bronner-Fraser & Fraser, 1988; Bronner-Fraser & Fraser, 1989). Clonal analysis revealed that an individual NCC can give rise to multiple cell types (Bronner-Fraser & Fraser, 1988; Bronner-Fraser & Fraser, 1989; Frank & Sanes, 1991). In contrast, the second hypothesis claims that NCCs are already fate-specified in the neural tube and prior to delamination (Krispin *et al.*, 2010). NCCs emigrate from the neural tube in a sequential manner to generate NCC derivatives in a ventral-to-dorsal order. That is sympathetic ganglia before dorsal root ganglia followed by melanocytes.

The migratory pathway of NCCs is determined by differentially expressed permissive and inhibitory guidance cues. As a result NCC migration occurs in a highly precise and coordinated manner. Guidance of NCCs seems to be largely determined by repulsive molecules, as permissive cues such as fibronectin and laminin are uniformly present. Non-permissive molecules expressed in the caudal half of the somite, such as ephrins (Krull *et al.*, 1997), F-spondin (Debby-Brafman *et al.*, 1999), versican V0 and V1 (Dutt *et al.*, 2006) and semaphorin 3A (Sema3A) (Eickholt *et al.*, 1999) form an inhibitory barrier for NCCs. Consequently NCCs only migrate through the rostral half of the somite. This specific migration pattern results in the characteristic segmentation of the DRGs and sympathetic ganglia (Dutt *et al.*, 2006) (see Fig. 2C).

The last NCCs giving rise to DRG cells emigrate at stage HH17 to HH18 following aggregation and condensation into nascent DRGs at stage HH20 (Frank & Sanes, 1991; Krispin *et al.*, 2010; Lallier & Bronner-Fraser, 1988; Rifkin *et al.*, 2000). Cells in the DRGs proliferate to generate glia and neurons of different subtypes, such as nociceptive, proprioceptive and mechanoceptive neurons. Coinciding with the peak of neurogenesis around HH24/HH25 the majority of neurons express neurotrophin tyrosine receptor kinase C (TrkC) with some subsets of neurons coexpressing TrkA or TrkB (Rifkin *et al.*, 2000). At around stage HH29 and coinciding with peripheral target innervation, a segregation of subpopulation-specific sensory neurons expressing only one Trk family member can be observed (Rifkin *et al.*,

2000). Small-diameter, unmyelinated nociceptive neurons expressing TrkA are localized to the dorsomedial part of the DRG whereas large-diameter, myelinated proprioceptive neurons expressing TrkC and mechanoceptive neurons expressing TrkB are restricted to the ventrolateral region of the DRG (Eide & Glover, 1997; Rifkin *et al.*, 2000) (see Fig. 3). The survival of the different sensory subtypes depends on specific neurotrophic factors supplied by the peripheral target organs and the expression of the respective Trk receptor family member. $TrkA^+$-nociceptive neurons, which mediate pain (nociception) and temperature sensation require nerve growth factor (NGF) for survival (Klein, 1994; Ruit *et al.*, 1992; Snider, 1994). $TrkC^+$-proprioceptive neurons sensing limb movement and position are NGF-independent but require neurotrophin-3 (NT-3) to survive (Ernfors *et al.*, 1994; Klein, 1994; Lamballe *et al.*, 1991; Ruit *et al.*, 1992; Snider, 1994; Tessarollo *et al.*, 1994). The third population of sensory subtypes contributes to $TrkB^+$-mechanoceptive neurons which respond to tactile stimuli. They largely depend on brain-derived neurotrophic factor (BDNF) (Klein, 1994; Ruit *et al.*, 1992; Snider, 1994; Soppet *et al.*, 1991; Squinto *et al.*, 1991).

Guidance of primary sensory afferents

Shortly after condensation into DRGs, the cell bodies of sensory neurons start to extend axons from the DRGs towards the dorsal spinal cord and into the periphery thereby bridging the central and peripheral nervous system. The first afferents reach the dorsal root entry zone (DREZ) of the lumbosacral spinal cord around day E3 to E4 (HH19 to HH23) in the chicken (Davis *et al.*, 1989; Eide & Glover, 1995; Perrin *et al.*, 2001) (Fig. 2A). Thereby, the axons fasciculate to form straight, parallel bundles, the so-called dorsal roots. The Ig-superfamily cell adhesion molecule (IgSF-CAM) F11/contactin is involved in proper fasciculation of sensory axons during their growth towards the DREZ (Perrin *et al.*, 2001). After entering the spinal cord, sensory afferents bifurcate in a Y- or T-shaped manner to run as a single bundle rostrally and caudally along the longitudinal axis of the dorsal spinal cord (Eide & Glover, 1995) (Fig. 2B, C). Proper guidance and bifurcation of sensory afferents in the dorsal spinal cord was found to be mediated by F11/contactin. Perturbation of F11/contactin changed the morphology of the dorsal root entry zone (DREZ) due to aberrant bifurcation of sensory afferents (Perrin *et al.*, 2001). Another study implicated Slits and the IgSF-CAM receptors, the Robos, in controlling the bifurcation of sensory axons in mice (Le Ma &

Tessier-Lavigne, 2007). In Slit1/Slit2 or Robo1/Robo2 double-mutant mice branches were leaving the DREZ and misprojected into the grey matter towards the midline of the spinal cord (Le Ma & Tessier-Lavigne, 2007).

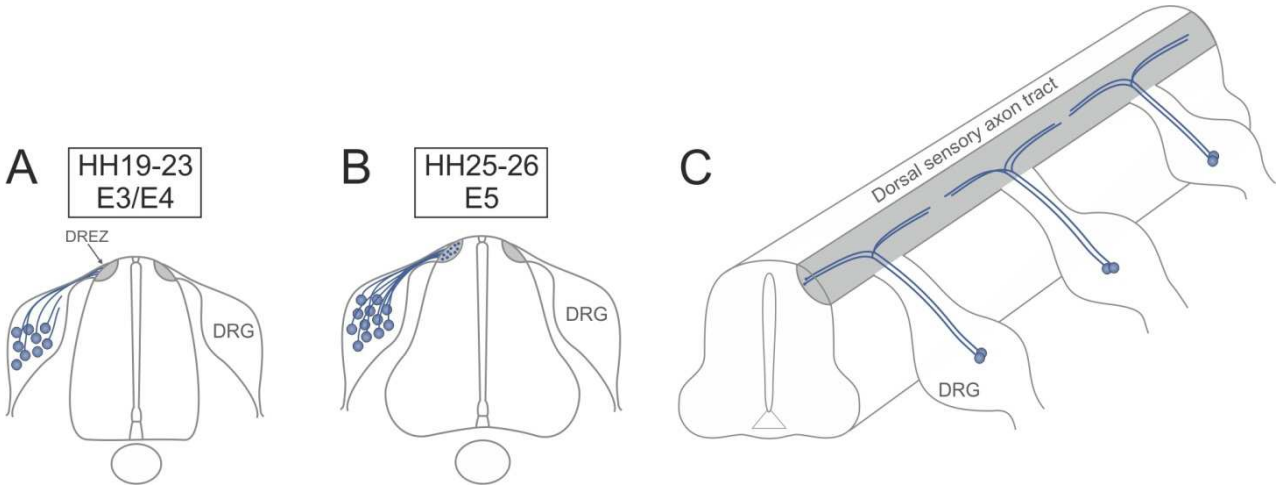

Figure 2. Pathway of primary sensory afferents. (A) Between HH19 and HH23 sensory axons start extending from the DRG and enter the dorsal spinal cord through the dorsal root entry zone (DREZ). (B, C) By stage HH25/26 axons have reached the DREZ (B) and bifurcate to elongate along the longitudinal axis as a fascicle, thereby forming the sensory axon bundle (grey) in the dorsal spinal cord (C). Note the segmented arrangement of DRGs along the spinal cord in (C). (A) and (B) represent schematic drawings of spinal cord and DRG cross sections and (C) represents a schematic drawing of the longitudinal spinal cord and DRGs.

The dorsal sensory axon bundle extends over several segments (Eide & Glover, 1995). However, collaterals branching off primary sensory afferents do not innervate the grey matter of the spinal cord before stage HH29 (E6) (Davis *et al.*, 1989; Lee *et al.*, 1988). A so-called waiting period precedes the invasion into target regions in the grey matter. During this waiting period Sema3A was found to be transiently expressed in the whole spinal cord including the region around the DREZ (Fu *et al.*, 2000). Repulsive Sema3A was suggested to keep primary sensory afferents from invading the grey matter and thereby steering them along the longitudinal axis in a bundle (Puschel *et al.*, 1996; Shepherd *et al.*, 1997). Formation of the longitudinal bundle may be supported by fasciculation of sensory axons by axonin-1, NgCAM and NrCAM interactions (Kunz *et al.*, 1998; Shiga *et al.*, 1997). This hypothesis has been supported by the finding that perturbation of axonin-1 in vivo resulted in a premature invasion of the dorsal grey matter (Perrin *et al.*, 2001). Together, the guidance of sensory afferents along the longitudinal axis and their exclusion from the grey matter during the waiting period is regulated by a combination of attractive forces derived

from adhesion molecules mediating fasciculation and repellent forces originating from cells located in the grey matter of the spinal cord (Perrin *et al.*, 2001).

Formation of sensory collaterals

Sensory collaterals start to penetrate the grey matter of the spinal cord at HH29 and continue to form synapses up to stage HH39 (Davis *et al.*, 1989; Lee *et al.*, 1988) (Fig. 3). It has been proposed that the end of the waiting period might be caused by a shift in the preference of sensory axons to extend in the grey matter compared to the dorsal axon bundle. Axonin-1, NgCAM and NrCAM have been implicated to regulate the preference of sensory afferents for each other (Shiga *et al.*, 1997). Changes in the expression levels of these cell adhesion molecules would lead to a weakened fasciculation among axons which might contribute to the invasion of sensory collaterals into the grey matter of the spinal cord (Shiga *et al.*, 1997). In line with this is the loss of Sema3A expression in the dorsal horn and its restriction to the ventral spinal cord at the time of collateral invasion (Fu *et al.*, 2000; Messersmith *et al.*, 1995). Through its receptor neuropilin1 (NRP1), Sema3A exerts a repulsive effect on NGF-dependent nociceptive collaterals (Pond *et al.*, 2002; Reza *et al.*, 1999). Thus, they are kept in the dorsal horn of the spinal cord where they innervate cells located in laminae I and II (Eide & Glover, 1997; Perrin *et al.*, 2001; Ruit *et al.*, 1992) (Fig. 3). In contrast, NT-3-dependent proprioceptive collaterals lose their sensitivity to Sema3A as NRP1 is downregulated (Fu *et al.*, 2000; Pond *et al.*, 2002; Reza *et al.*, 1999; Shepherd *et al.*, 1997). Hence, proprioceptive fibers are allowed to innervate the motoneurons located in the ventral horn and neurons in the intermediate zone (Eide & Glover, 1997; Perrin *et al.*, 2001; Ruit *et al.*, 1992) (Fig. 3). In addition, the attractive Ig-superfamily members axonin-1 and F11/contactin have also been implicated in the subpopulation-specific pathfinding of sensory collaterals (Perrin *et al.*, 2001). A selective guidance effect of axonin-1 together with its binding partner NgCAM could be observed on nociceptive collaterals whereas F11/contactin interaction with NrCAM influence proper pathfinding of proprioceptive collaterals (Perrin *et al.*, 2001). Thus, the pathway of nociceptive and proprioceptive collaterals gets segregated by selective inhibition via NRP1-Sema3A interaction as well as guidance by attractive cues allowing modality-specific target innervation.

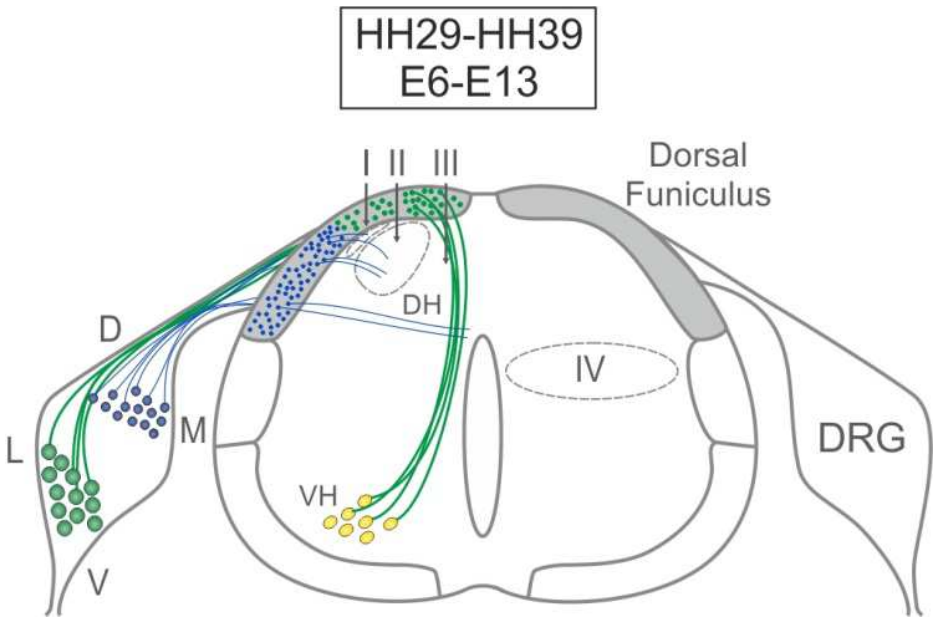

Figure 3. Central projections of sensory axons. Sensory collaterals project to different targets in the spinal cord grey matter depending on their neuronal subtype. Nociceptive axons (blue) residing in the lateral dorsal funiculus (grey) extend collaterals (blue) into laminae I and II of the dorsal horn (DH). Some nociceptive collaterals extend to cells near the ventricular zone. Proprioceptive axons (green), restricted to the medial dorsal funiculus (grey), send collaterals (green) to the ventral horn where they from synapses with motoneurons (yellow). Note the subpopulation-specific locations of nociceptive and proprioceptive neurons in the dorsomedial and ventrolateral DRG, respectively.

Sensory neural circuit formation depends on members of the IgCAM-superfamily

Molecules of the Ig-superfamily of cell adhesion molecules are involved in many steps during the formation of sensory neural circuits, from the time point of primary sensory axon extension to the DREZ to sensory collateral formation. As described above, F11/contactin mediates fasciculation between sensory axons leading to the formation of dorsal roots. Furthermore, F11/contactin, axonin1, NrCAM, NgCAM and Robo regulate bifurcation of primary afferents and extension along the longitudinal axis as a tight fascicle. At later stages, the differential interactions between axonin1 and NgCAM and F11/contactin and NrCAM guide sensory collaterals in a subtype-specific manner to their correct targets in the grey matter of the spinal cord. Thus, the combined action of different IgSF-CAMs ensures and specifies the pathfinding of sensory axons in every step of neural circuit formation. Interestingly, some of these molecules are even important later in development, namely in synaptogenesis (Bukalo & Dityatev, 2012; Missler *et al.*, 2012). Taken together, this highlights the importance of cell adhesion molecules throughout the development of the nervous system and raises the question, which other IgSF-CAMs could be involved in the formation of neural circuits.

Review: Synaptic cell adhesion molecules – A link between neural circuit formation and neurodevelopmental diseases

Synaptic Cell Adhesion Molecules –

A Link between Neural Circuit Formation and Neurodevelopmental Diseases

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Abstract

Synaptic plasticity is the key feature of brain function. Learning and memory would not be possible without it. Not surprisingly, compromised synapse function and synaptic plasticity are implicated in a variety of neural disorders. Therefore, an understanding of the molecular mechanisms of synaptic plasticity would be instrumental for our comprehension of the pathogenesis of neural disorders, such as schizophrenia, autism spectrum disorders, or intellectual disability. Obviously, synaptic plasticity requires the formation of functional synapses. Therefore, synaptogenesis and synaptic plasticity are often linked together. Indeed a variety of molecules that have been identified in synaptogenesis have also been implicated in synaptic plasticity and vice versa. In particular, synaptic cell adhesion molecules have obtained a lot of attention, as they were shown to be sufficient for synapse induction but also crucial for synaptic plasticity. In this review, we summarize the role of synaptic cell adhesion molecules in synaptogenesis and synaptic plasticity but take the family of SynCAMs as an example to point out that synaptic cell adhesion molecules are involved in more than synaptogenesis during development. In fact, SynCAMs are important contributors to axon guidance and, therefore, affect already early aspects of neural circuit formation.

Introduction

Neurodevelopmental disorders, including autism spectrum disorders (ASD), intellectual disability (ID), and schizophrenia, but also rare syndromes, such as Joubert syndrome (Doherty, 2009; Joubert *et al.*, 1968) have attracted a lot of attention during the last decade. Although the etiology of these disorders is unknown in most cases, the focus was, and largely still is, on aberrant synaptic function. Both deficits in synaptogenesis and synaptic plasticity have been implicated in the pathogenesis of these disorders. However, one has to keep in mind that synaptic dysfunction may not be the only problem in aberrant neural circuit function. Errors in axonal targeting may contribute to neural circuit malfunction in several ways. Firstly, aberrant connectivity may prevent efficient signal transduction to the appropriate target or effector cell. Secondly, aberrant axon guidance may result in excessive cell death due to the lack of neurotrophic support, and therefore prevent effective signal transduction. Unfortunately, we do not have the technology that would allow us to study axonal connections at high resolution in the human brain in vivo. Thus, aberrant axonal connections can only be detected when changes are massive and when they affect major fiber tracts. An example has been described for Joubert syndrome, where DTI (diffusion tensor imaging) studies provided sufficient resolution to detect aberrant brain wiring in affected individuals (Engle, 2010; Juric-Sekhar *et al.*, 2012; Poretti *et al.*, 2007). Joubert syndrome is a rare autosomal recessive disease causing intellectual disability, autism, breathing anomalies, ataxia, and hypotonia (Doherty, 2009; Joubert *et al.*, 1968). The hallmark diagnostic feature of the disease is the appearance of the so-called ‘molar tooth sign’ in magnetic resonance images caused by hypoplasia of the cerebellar vermis (Maria *et al.*, 1999).

The term ASD defines a heterogeneous group of neurodevelopmental diseases which have in common that affected individuals have deficits in social communication, impaired use of language and stereotyped repetitive behaviors (Amaral *et al.*, 2008). In addition to these core symptoms up to 70% of the patients diagnosed with ASD suffer from ID and about 25% from epileptic seizures (Amaral *et al.*, 2008; Tuchman & Rapin, 2002; Zoghbi & Bear, 2012). The etiology of autism is complex but not fully understood. More than in other neurodevelopmental disorders, aberrant connectivity has been considered in the pathogenesis of ASD. A variety of studies have concluded that underconnectivity contributes

to the pathology of ASD (Frith, 2004; Geschwind & Levitt, 2007; Hughes, 2007), although some studies led authors to the conclusion that ASD is associated with hyperplasticity and inappropriate synaptic consolidation, which was linked to the common feature of savant abilities in about 10% of autistic persons (Kelleher & Bear, 2008). In support of the hypothesis that ASD and some forms of ID are due to deficits in axon guidance have come from genome-wide association and linkage studies (for references see Stoeckli, 2012). Still, the major focus has been put on those genes which are involved in synaptic structure, function and plasticity.

Among the "disease genes" scaffold molecules, such as SHANK3, are found as top hits on the lists (Uchino & Waga, 2013). In addition, mutations in synaptic cell adhesion molecules have been identified in patients diagnosed with ASD and ID. Identification of rare mutations and copy number variations (CNV) in genes encoding neuroligins (NLGNs) and neurexins (NRXs) in individuals with non-syndromic autism have directed the focus to synaptic cell adhesion molecules in the etiology of ASD (Sudhof, 2008). Mutations in the neuroligin-neurexin-system have been associated with an imbalance in excitatory and inhibitory synaptic transmission as an underlying cause of ASD, highlighting the central role of these proteins in organizing excitatory and inhibitory synapses (Bourgeron, 2009). Recently, two missense mutations in the synaptic cell adhesion molecule 1 (SynCAM1) have been reported in ASD patients (Zhiling *et al.*, 2008). Synaptic cell adhesion molecules not only bring together and stabilize synapses but they also play an important role in synapse function. Therefore malfunction of synaptic cell adhesion molecules are linked to ASD, ID and other neurological diseases.

In addition to mutations in genes encoding synaptic scaffold molecules and synaptic cell adhesion molecules, highly penetrant mutations in genes affecting synaptic signaling and protein transcription have been identified as causes of syndromic forms of autism and ID. Among those are mutations in the FMR1 gene causing Fragile X syndrome, MECP2 causing Rett syndrome, TSC1/TSC2 underlying tuberous sclerosis, PTEN linked to hamartoma-tumor syndrome including macrocephaly, NF1 linked to neurofibromatosis and UBE3A implicated in Angelman syndrome (Ebert & Greenberg, 2013; Kelleher & Bear, 2008; Zoghbi & Bear, 2012). Interestingly, all of these genes are involved in the activity-dependent regulation of mRNA translation (i.e. FMR1, TSC1/TSC2, PTEN, NF1 via mTOR/Ras-ERK pathway), gene

transcription (i.e. MECP2 via binding to methylated cystosines and repressing transcription), or ubiquitination and degradation (i.e. UBE3A) of synaptic proteins. Hence, disruptive mutations in these genes are suggested to alter synaptic protein levels causing synaptic dysfunction, which in turn was thought to be the major cause of ASD.

Although autism has a strong genetic component, with a concordance rate of 70-90% for monozygotic twins compared to less than 10% for dizygotic twins (Zoghbi, 2003), the number of genes associated with ASD only explain a small minority of the cases. Furthermore, the contribution of environmental factors to the etiology of ASD is difficult to investigate (Persico & Bourgeron, 2006).

Synaptic cell adhesion molecules

Synaptic cell adhesion molecules have multiple functions at the synapse. They are involved in the formation, function, organization, specification, maintenance and activity-dependent plasticity of synapses (Bukalo & Dityatev, 2012; Missler *et al.*, 2012). In the following paragraph we summarize current knowledge about the role of synaptic cell adhesion molecules in synaptogenesis and synaptic plasticity but also highlight their involvement in earlier processes of neural circuit formation. We further comment on their contribution to the pathogenesis of ASD and other neurodevelopmental diseases. A special focus is laid on the synaptic cell adhesion molecules of the SynCAM family.

Neuroligins and Neurexins:

Postsynaptic neuroligins (NLGs) and their presynaptic binding partners, the neurexins (NRXs), are trans-synaptic cell adhesion molecules (Dean *et al.*, 2003; Song *et al.*, 1999) (Fig. 1). In humans, five neuroligin (NLG1, NLG2, NLG3, NLG4X and NLG4Y) and three neurexin genes have been identified (Craig & Kang, 2007). Neurexins exist as longer α-NRXs and shorter β-NRXs due to two alternative promoters. Both neurexins and, to a lesser extent, neuroligins are subject to alternative splicing which generates a large diversity of isoforms with different binding affinities (Boucard *et al.*, 2005; Chih *et al.*, 2006; Graf *et al.*, 2006; Tabuchi & Sudhof, 2002).

The synaptogenic effect of the neuroligin-neurexin complex was initially discovered in heterologous co-culture assays. Overexpression of neuroligins and neurexins in non-neuronal cells triggered the differentiation of pre- and postsynaptic specializations in axons (Graf *et al.*, 2004; Scheiffele *et al.*, 2000). Overexpression of neuroligins in cultured neurons increased the number of synapses, whereas knockdown by RNAi reduced the number of synapses indicating that neuroligins induce synapse formation (Chih *et al.*, 2004; Chih *et al.*, 2005; Dean *et al.*, 2003; Sara *et al.*, 2005). Not entirely in line with these findings are data obtained from in vivo studies with knockout mice for NLG1, NLG2 and NLG3 and α-NRXs. Triple neuroligin knockout mice die at birth due to respiratory failure (Varoqueaux *et al.*, 2006). They show impairments in synaptic transmission, mainly at inhibitory synapses, but normal synapse numbers. Deletions of α-NRXs are lethal due to dysfunction of presynaptic voltage-gated Ca^{2+}-channels resulting in neurotransmitter release deficits (Missler *et al.*, 2003). However, there are no changes in synapse number in α-NRX triple knockout mice. The findings of the knockout studies suggest a role of neuroligins and neurexins in synaptic function rather than the initial formation of synapses (Sudhof, 2008; Varoqueaux *et al.*, 2006).

It has been shown that NLG1 is localized to excitatory whereas NLG2 is present at inhibitory synapses (Chih *et al.*, 2005; Song *et al.*, 1999; Varoqueaux *et al.*, 2004). NLG3 is localized to both types of synapses (Budreck & Scheiffele, 2007). The differential localization of the different neuroligin family members suggests a role in specification of excitatory and inhibitory synapses. Indeed, overexpression of NLG1 in cultured neurons increased EPSCs but not inhibitory synaptic responses whereas NLG2 overexpression increased IPSCs without affecting excitatory responses (Chubykin *et al.*, 2007). The effects of NLG1 and NLG2 can be reversed by chronic inhibition of NMDA signaling or inhibition of AMPA-receptors (AMPAR) and $GABA_A$-receptors ($GABA_AR$), respectively, suggesting that neuroligins specify synapses in an activity-dependent manner. In agreement with these findings, deletion of NLG1 decreases the amplitude of NMDA receptor (NMDAR)-mediated EPSCs and deletion of NLG2 results in reduced IPSC amplitudes (Chubykin *et al.*, 2007). Furthermore, NLG1 and NLG2 specify postsynaptic identities by recruiting different adaptor proteins. Endogenous NLG1, NLG3 and NLG4 colocalize with PSD95, a scaffold protein at glutamatergic synapses, whereas NLG2 coclusters with gephyrin, a scaffold protein specific for inhibitory synapses (Graf *et al.*, 2004; Song *et al.*, 1999) (Fig. 1). By specifying excitatory and inhibitory synapses, neuroligins

contribute to the determination of the balance between excitation and inhibition and, thus, to proper neural circuit function. Neuroligins together with PSD95 play a critical role in controlling this balance (Levinson *et al.*, 2005; Prange *et al.*, 2004). Overexpression of PSD95 recruits NLG1 to excitatory synapses, thereby enhancing their maturation (Levinson *et al.*, 2005; Prange *et al.*, 2004). Furthermore, the distribution of NLG2 is shifted from inhibitory to excitatory synapses when PSD95 is overexpressed (Graf *et al.*, 2004; Levinson *et al.*, 2005). This shows that the level of PSD95 controls the excitatory to inhibitory (E/I) ratio by modulating the localization and retention of neuroligins to excitatory and inhibitory synapses, respectively (Graf *et al.*, 2004; Levinson *et al.*, 2005; Prange *et al.*, 2004).

Interestingly, disturbances in the balance between excitatory and inhibitory synaptic currents have been associated with neurodevelopmental and psychiatric disorders (Bourgeron, 2009; Rubenstein & Merzenich, 2003). In line with this, mutations in NRX1, NLG1, NLG3 and NLG4 genes have been discovered in patients with ASD, intellectual disability and schizophrenia (Jamain *et al.*, 2003; Kim *et al.*, 2008a; Kirov *et al.*, 2008; Laumonnier *et al.*, 2004; Rujescu *et al.*, 2009; Yan *et al.*, 2005). Several of the neuroligin and neurexin mutations found in autistic patients have been introduced to mice. Mice carrying the NLG3 gain-of-function mutation R451C show deficits in social interactions, thus, mimicking a key feature of autism (Tabuchi *et al.*, 2007). At the physiological level, an increase in inhibitory synaptic transmission was found in these mice. Similarly, loss-of-function mutation in the NLG4 gene leads to impairments in social behavior and ultrasonic communication (Jamain *et al.*, 2008), reflecting some behavioral phenotypes observed in patients suffering from autism. Mice lacking α-NRX1 display reduced mEPSC frequencies and a decrease in excitatory synaptic strength but no alterations in inhibitory synaptic currents (Etherton *et al.*, 2009).

Taken together, neuroligins and their presynaptic partners the neurexins play critical roles in synapse function and maturation by specifying excitatory and inhibitory synapses in an activity-dependent manner (Chubykin *et al.*, 2007). Disruption of neuroligin and neurexin genes results in alterations in excitatory and inhibitory synaptic transmission and consequently in an imbalance of the E/I ratio, which is associated with the etiology of ASD. The fact that a subset of autistic patients suffer from seizures supports the role of neurexins

and neuroligins in controlling the E/I ratio, as such imbalances increase the risk for epilepsy (Rubenstein & Merzenich, 2003).

Leucin-rich repeat transmembrane neuronal proteins (LRRTMs):

Leucin-rich repeat transmembrane neuronal proteins (LRRTMs) have been identified as a new gene family with four members (LRRTM1-4) that belong to the leucin-rich repeat (LRR) superfamily (Lauren *et al.*, 2003). They are predominantly expressed in neurons in the central nervous system (CNS) during development but also in mature brains (Lauren *et al.*, 2003). LRRTMs have been validated as synaptogenic proteins as they induce glutamatergic presynaptic differentiation in the heterologous co-culture assay. LRRTM1 and LRRTM2 have the most potent synaptogenic effect (Ko *et al.*, 2009; Linhoff *et al.*, 2009; Wit *et al.*, 2009). In line with a role in excitatory synaptogenesis, overexpression and knockdown of LRRTM2 in hippocampal neurons results in a concomitant increase and decrease, respectively, in excitatory synaptic density (Ko *et al.*, 2009; Wit *et al.*, 2009). LRRTM2 additionally acts as postsynaptic organizer, as it interacts with PSD95 and contributes to excitatory synaptic transmission via regulation of AMPAR surface expression (Linhoff *et al.*, 2009; Wit *et al.*, 2009) (Fig. 1). LRRTMs do not interact homophilically. As presynaptic trans-interaction partners for LRRTM2, NRX1α and β have been identified (Ko *et al.*, 2009; Siddiqui *et al.*, 2010; Wit *et al.*, 2009) (Fig. 1). Knockdown of NRX1 prevents the presynapse-inducing activity of LRRTM2 showing that LRRTM2 and NRX1 are necessary for excitatory synapse development (Wit *et al.*, 2009). Interestingly, the neurexin binding site of LRRTMs and neuroligins is overlapping, thus, the two proteins cannot bind simultaneously (Ko *et al.*, 2009; Siddiqui *et al.*, 2010). Although LRRTM and neuroligins compete for the binding of neurexins, they exert similar functions and cooperate in an additive manner in the promotion of glutamatergic synapses during development (Siddiqui *et al.*, 2010; Soler-Llavina *et al.*, 2011). In contrast, in mature synapses LRRTMs and neuroligins seem to play divergent roles. Whereas knockdown of NLG1 in mature hippocampal slices results in a significantly reduced NMDAR/AMPAR ratio due to decreased NMDAR-dependent transmission, knockdown of LRRTM1 and LRRTM2 does not have any effect on synaptic transmission (Soler-Llavina *et al.*, 2011). However, this does not imply that LRRTMs do not have any function in the mature brain. Recently, it has been shown knockdown of LRRTM1,

LRRTM2 and NLG3 in a NLG1-knockout background results in loss of excitatory synapses. This synapse loss is prevented by blocking synaptic activity, Ca^{2+}-influx or Ca^{2+}/calmodulin (CaM)-kinase indicating that LRRTMs and neuroligins have a signaling function that is necessary to maintain excitatory synapses and to prevent activity- and Ca^{2+}/calmodulin-dependent synapse elimination (Ko *et al.*, 2011).

Dysfunction of LRRTM1 and NRX1 has been implicated as possible cause of schizophrenia (Francks *et al.*, 2007; Kirov *et al.*, 2008; Rujescu *et al.*, 2009). Taking into account that NRX1 and LRRTMs act together in synapse formation, one could speculate that mutations in the neurexin-LRRTM pathway are involved in the etiology of schizophrenia and probably also in other cognitive diseases.

N-Cadherins:

Classical cell adhesion molecules (CAMs) at synapses are cadherins, which link pre- and postsynaptic elements by homophilic, Ca^{2+}-dependent interaction and through their intracellular binding partners, the catenins (Geiger & Ayalon, 1992; Takeichi & Abe, 2005) (Fig.1). After initial axo-dendritic contacts N-cadherins accumulate at active zones and postsynaptic densities of nascent synapses. During maturation of synapses, N-cadherins get restricted to sites surrounding synapses, the puncta adherentia junctions (Benson & Tanaka, 1998; Takeichi & Abe, 2005). The role of N-cadherins in establishing synaptic contacts is supported by the fact that disruption of cadherin-based adhesion inhibits the formation of synapses (Bozdagi *et al.*, 2004). However, N-cadherins are not sufficient for synapse induction, as its overexpression in non-neuronal cells does not trigger presynaptic development in contacting axons (Sara *et al.*, 2005). Rather than having an inductive role in synapse formation, N-cadherins on the one hand provide structural support by controlling spine morphology and stabilizing synapses (Togashi *et al.*, 2002) and on the other hand impact synaptic plasticity in developing and mature synapses. Neuronal activity enhances expression of N-cadherin in spines leading to stabilization of dendritic spines and, as a consequence, long-term potentiation (LTP) (Bozdagi *et al.*, 2010; Mendez *et al.*, 2010). Interfering with N-cadherin function abolishes plasticity-mediated stabilization of dendritic spines (Mendez *et al.*, 2010) and reduces LTP and LTP-induced enlargement of dendritic spines (Bozdagi *et al.*, 2010). Hence, N-cadherin adhesion provides long-term synapse

stability and coordinates structural and functional synaptic plasticity. Besides long-term plasticity, N-cadherins have been implicated in short-term plasticity at glutamatergic synapses (Jungling *et al.*, 2006). N-cadherin deficient synapses showed impairments in presynaptic vesicle exocytosis under enhanced neuronal activity and, as a consequence, defects in short-term synaptic plasticity indicated by an increase in short-term depression. Furthermore, the findings in this study showed that N-cadherin trans-synaptically controls short-term synaptic plasticity in a retrograde manner (Jungling *et al.*, 2006).

Although N-cadherins are not able to induce de novo synapse formation, they have been implicated in regulating the formation of excitatory synapses in cooperation with NLG1 through a common functional pathway (Aiga *et al.*, 2011) (Fig. 1). In this study, the authors could show that N-cadherin clustering in early developing neurons precedes clustering of NLG1. Overexpression of N-cadherin enhanced clustering of NLG1 thereby inducing formation of synapses. The loss of synapse density after knockdown of NLG1 could be partially rescued by overexpressing N-cadherin, due to recruitment of NLG2 to N-cadherin clusters. Another study shows that N-cadherin's ability to control presynaptic vesicle clustering (Bamji *et al.*, 2003; Bozdagi *et al.*, 2004; Jungling *et al.*, 2006; Togashi *et al.*, 2002) is mediated by recruiting and activating the NLG1 system through the scaffold protein S-SCAM (Stan *et al.*, 2010). The cooperation between N-cadherin and neuroligin adhesion systems play important roles in the development of synapses as they link the function of N-cadherin in establishing and stabilizing contacts to the function of neuroligins in specifying synapse identities (Chubykin *et al.*, 2007; Graf *et al.*, 2004).

As it has been shown for the neuroligin and neurexin family, mutations in cadherin genes have been associated with susceptibility to ASD (Wang *et al.*, 2009). Six single nucleotide polymorphisms (SNPs) in cadherin9 and cadherin10 have been found in a genome-wide association study for autism. Disruption of cadherin-mediated adhesion could imply structural and functional disconnection of synapses. Abnormal connectivity or disconnection of higher brain regions during development is discussed as one of multiple causes of autism (Frith, 2004; Geschwind & Levitt, 2007; Hughes, 2007). Furthermore, Wang and colleagues focused on the integration of ASD-linked mutations in various cell adhesion molecules and found a strong association of cadherin/neurexin genes suggesting a collective contribution of these cell adhesion molecules to ASD (Wang *et al.*, 2009).

Cell adhesion molecules of the immunoglobulin-superfamily

Another family of classical cell adhesion molecules is the immunoglobulin (Ig)-superfamily of cell adhesion molecules (IgSF-CAMs). In contrast to cadherins, their interaction is Ca^{2+}-independent and more complex, as in addition to homophilic also heterophilic interactions are common. More than 100 IgSF-CAMs have been identified. Based on structural features, they are subdivided into subfamilies. Many of them have been studied in neural development, predominantly in axon guidance (Stoeckli, 2004).

L1 subfamily of IgSF-CAMs:

L1/NgCAM and NrCAM (NgCAM-related cell adhesion molecule) together with CHL1 (Close homolog of L1) and neurofascin form the L1 subgroup of the IgSF-CAMs. They consist of six immunoglobulin (Ig)-like and five fibronectin-type III-domains (Hortsch, 1996). L1/NgCAM was one of the first IgSF-CAMs identified as cause for cognitive impairments (Wong *et al.*, 1995; Fransen *et al.*, 1995). A variety of L1 mutations were found in boys with intellectual disability/mental retardation, ataxia, shuffling gait, adducted thumbs and hydrocephalus, summarized as MASA (Mental retardation, Adducted thumbs, Spastic paraplegia and Aphasia) or CRASH (Corpus callosum hypoplasia, Retardation, Adducted thumbs, Spastic paraplegia and Hydrocephalus) syndrome (Fransen *et al.*, 1995). Mice lacking L1 showed much milder deficits (Dahme *et al.*, 1997) compared to the human phenotype. Hydrocephalus was found to be strain dependent and no obvious cognitive impairment was observed. However, in some areas of the nervous system, axon guidance defects were found. For instance, the decussation of the corticospinal tract did not form in L1 knockout mice (Cohen *et al.*, 1998b).

Another member of the L1 subfamily is NrCAM. This cell adhesion molecule is exclusively expressed in the nervous system in neurons, Schwann cells and in floor-plate cells of the spinal cord (Grumet, 1997). Expression persists in the mature nervous system which is in contrast to L1/NgCAM whose expression level decreases in the adult brain (Grumet, 1992, 1997). NrCAM can interact in a homophilic and heterophilic manner, although the homophilic interactions are weak (Mauro *et al.*, 1992). The heterophilic interaction between NrCAM and the contactin family member CNTN2/TAG1/axonin1 has been shown to be

crucial for the contact-mediated guidance of commissural axons across the midline, the floor plate, of the chicken spinal cord (Stoeckli & Landmesser, 1995; Stoeckli *et al.*, 1997). The same interaction has been implicated in the promotion of neurite outgrowth from chicken dorsal root ganglia (DRGs) (Lustig *et al.*, 1999) and in the initial contact of sensory axons with Schwann cells necessary for myelination (Suter *et al.*, 1995). Besides commissural axon guidance and outgrowth of DRG axons, NrCAM interacting with another contactin family member, CNTN1/F3/F11, is required for pathfinding of proprioceptive collaterals to the ventral horn of the chicken spinal cord (Perrin *et al.*, 2001) as well as for neurite outgrowth of chicken tectal cells (Morales *et al.*, 1993; Volkmer *et al.*, 1996). Later in development, NrCAM accumulates with neurofascin and ankyrins, a family of spectrin-binding molecules, at the node of Ranvier (Davis *et al.*, 1996). The high NrCAM expression levels in adult brains and its interaction with ankyrins suggested a role in the stabilization of cell-cell contacts and recruitment of postsynaptic scaffold components, as it has been found for neural cell adhesion molecule (NCAM) (Davis *et al.*, 1996; Sytnyk *et al.*, 2006). Moreover, the cytoplasmic tail of NrCAM is able to bind to PDZ-domain containing proteins, such as PSD95, which are important for clustering of receptors and channels of excitatory postsynapses (Grumet, 1997; Hung & Sheng, 2002). These findings indicate that, in addition to its role in axon elongation and guidance, NrCAM might play a role in synaptogenesis. A function of NrCAM throughout neural circuit formation would be in line with the identification of this cell adhesion molecule as a susceptibility gene of ASD (Bonora *et al.*, 2005). However, the association of NrCAM with autism is still debated (Hutcheson *et al.*, 2004).

Contactins:

Contactins (CNTNs) form another subgroup of IgSF-CAMs that are exclusively expressed in the nervous system (Shimoda & Watanabe, 2009). The contactin family consists of six members, CNTN1/F3 (mouse)/F11 (chick), CNTN2/TAG1 (mouse)/axonin1 (chick), CNTN3/BIG1, CNTN4/BIG2, CNTN5/NB2 and CNTN6/NB3. They typically contain six Ig-like domains and four fibronectin-type III-like domains and are linked to the plasma membrane via a GPI-anchor. Like the L1-subfamily, they can mediate homo- and heterophilic binding.

Among the contactin family members, CNTN1/F3/F11 and CNTN2/TAG1/axonin1 have been studied most intensively, particularly in axon guidance. Both, F11 and axonin1 have been

implicated in subtype-specific guidance of sensory axons from DRGs to their final targets in the grey matter of the chicken spinal cord (Perrin *et al.*, 2001). Perturbation of F11 by injecting function-blocking antibodies resulted in pathfinding errors of sensory afferents in the DREZ and failure of proprioceptive collaterals to extend to the ventral horn of the spinal cord. Absence of axonin1 led to premature invasion of sensory axons into the grey matter and pathfinding errors of nociceptive collaterals in the dorsal horn of the spinal cord. NrCAM and L1/NgCAM were identified as corresponding binding partners of F11 and axonin1, respectively (Perrin *et al.*, 2001). These findings show that F11 and axonin1 are required for the pathfinding of primary sensory afferents and collaterals, fasciculation of sensory afferents as well as neurite extension. Other studies have highlighted the involvement of interactions between axonin1 and NgCAM or NrCAM and F11 and NrCAM in the promotion of outgrowth and fasciculation of sensory neurites as well as mediation of neuron-glia contacts (Buchstaller *et al.*, 1996; Kuhn *et al.*, 1991; Kunz *et al.*, 1998; Lustig *et al.*, 1999; Morales *et al.*, 1993; Stoeckli *et al.*, 1996; Suter *et al.*, 1995). Besides this, the axonin1-NgCAM and the axonin1-NrCAM complex mediate different functions in the pathfinding of commissural axons to and across the floor plate, the first being essential for fasciculation of commissural axons and the latter being important for proper guidance at the midline (Stoeckli & Landmesser, 1995; Stoeckli *et al.*, 1997).

Besides these early roles of CNTN1/F3/F11 and CNTN2/TAG1/axonin1 in the development of the neuronal network, both have been implicated in myelination. CNTN1/F3/F11 localizes at the nodes of Ranvier of myelinated fibers where it associates with the β-subunit of sodium channels, thereby enhancing the expression of these channels in the axonal cell membrane (Kazarinova-Noyes *et al.*, 2001). At the paranodes CNTN1/F3/F11 is required for the correct targeting of Caspr, a member of the neurexin family, from the endoplasmic reticulum to the axonal membrane (Faivre-Sarrailh *et al.*, 2000). The interaction of this complex with neurofascin155, expressed by Schwann cells, is important for the proper formation of the paranodal junction (Charles *et al.*, 2002). At the juxtaparanodal region, Caspr2, also known as contactin associated protein-like 2 (CNTNAP2), associates with potassium channels and binds glial-expressed CNTN2/TAG1/axonin1 (Traka *et al.*, 2002; Traka *et al.*, 2003). This complex is required for the clustering of potassium channels at the juxtaparanode (Poliak *et al.*, 2003). CNTN1/F3/F11 and CNTN2/TAG1/axonin1 are also present at the synapse together with their respective binding partners Caspr and Caspr2/CNTNAP2 (Shimoda &

Watanabe, 2009). CNTN1/F3/F11 associates with Caspr and mediates its localization to the surface of CA1 pyramidal synapses (Murai *et al.*, 2002). This complex further recruits and stabilizes receptor protein tyrosine phosphatase β (RPTPβ). CA1 synapses of CNTN1-deficient mice exhibit impaired short-range and long-range synaptic plasticity, indicated by reduced paired-pulse facilitation and decreased NMDAR-dependent long-term depression (LTD). This shows that CNTN1/F3/F11 affects synaptic plasticity by stabilizing LTD (Murai *et al.*, 2002).

Much less is known about the other members of the contactin family, such as CNTN3/BIG1, CNTN4/BIG2, CNTN5/NB2 and CNTN6/NB3. CNTN4/BIG2 is strongly expressed in olfactory sensory neurons in the olfactory epithelium, where it acts as an axon guidance cue (Kaneko-Goto *et al.*, 2008). CNTN5/NB2 is prominently expressed in the auditory system. Disruption of the CNTN5/NB2 gene results in deficits of neuronal activity in the auditory system (Li *et al.*, 2003). CNTN6/NB3 expression is strong in the cerebellum, mainly in a subset of granule cells and in Purkinje cells. CNTN6-deficient mice suffer from impaired motor coordination (Takeda *et al.*, 2003). More interestingly, disruption of CNTN3/BIG1, CNTN4/BIG2, CNTN5/NB2 and CNTN6/NB3 have been implicated in ASD and single nucleotide polymorphism (SNP) of CNTN5/NB2 has been linked to schizophrenia (Glessner *et al.*, 2010; Morrow *et al.*, 2008; Roohi *et al.*, 2009; van Daalen *et al.*, 2011; Zuko *et al.*, 2013). For CNTN1/F3/F11 and CNTN2/TAG1/axonin1, no mutations associated with autism have been found so far. When considering the very early functions of these molecules in the wiring of the nervous system, it would not come as a surprise if deletions of these genes were lethal and, hence, could not be identified as disease-linked genes. However, rare homozygous mutations in Caspr2/CNTNAP2, the interaction partner of CNTN2 at the synapse and at juxtaparanodes, have been detected in patients with autism and seizures (Bakkaloglu *et al.*, 2008), suggesting that disruption of the contactin-pathway might be involved in the pathogenesis of ASD.

NCAM:

Neural cell adhesion molecule (NCAM) was discovered as one of the first molecules mediating homophilic and heterophilic adhesion between neurons (Cunningham *et al.*, 1987; Nielsen *et al.*, 2010). Adhesion of NCAM is regulated by polysialic acid (PSA) whose addition attenuates NCAM but also other interactions, such as L1/NgCAM mediated adhesion (Rutishauser, 1996). NCAM and PSA-NCAM play important roles in many processes during the development of neuronal networks, such as axon fasciculation, outgrowth promotion and axon guidance (Cremer *et al.*, 1997; Rutishauser, 1985; Rutishauser & Edelman, 1980) as well as neuronal migration (Tomasiewicz *et al.*, 1993). Furthermore, NCAM and PSA have been implicated in the innervation of muscle and the development of the neuromuscular junction (NMJ). NCAM mediates interactions between motor axons and myotubes and is essential for branching of axons (Landmesser *et al.*, 1988). Thereby PSA levels on NCAM regulate fasciculation of motor axons and thus intramuscular nerve branching patterns (Landmesser *et al.*, 1990). During initial development of the NMJ efficiency of synaptic transmission is very low, as motoneurons release acetylcholine from growth cones as well as axons and myotubes express acetylcholine receptors diffusely on their surface (Sanes & Lichtman, 1999). However, after contact the NMJ matures leading to clustering of synaptic vesicles to active zones and opposed clustering of acetylcholine receptors in the postsynapse. In mice lacking all three isoforms of NCAM (GPI-anchored 120 kDa and transmembrane 140 kDa and 180 kDa isoform) the immature release machinery is retained and coexists with the mature synaptic release system, which is more diffusely arranged (Polo-Parada *et al.*, 2001). This suggests that NCAM plays an important role in the molecular organization and maturation of the presynaptic terminal at the NMJ. Furthermore, NCAM knockout mice exhibit periods of total transmission failures in response to high-frequency repetitive stimulation (Polo-Parada *et al.*, 2001). The function of NCAM in the organization of the presynaptic release machinery, which ensures effective transmission with repetitive stimulation, was assigned to the C-terminus of the 180 kDa isoform, which binds to myosin light chain kinase (MLCK) (Polo-Parada *et al.*, 2004; Polo-Parada *et al.*, 2005).

NCAM is also essential for synapse formation, maturation and plasticity in the CNS. During early synaptogenesis, NCAM clusters at nascent synapses of cultured hippocampal neurons and stabilizes initial contacts by recruiting trans-Golgi network (TGN)-derived organelles

(Sytnyk *et al.*, 2002). NCAM also promotes the accumulation of spectrin, thereby assembling spectrin-based postsynaptic scaffold elements, such as NMDARs and Ca2+/calmodulin-dependent protein kinase II α (CamKIIα) (Sytnyk *et al.*, 2006). The recruitment of postsynaptic components to nascent synapses suggests a role for NCAM in modulating synaptic strength. Indeed, the synaptic strength of excitatory synapses is controlled by the expression of postsynaptic NCAM in an activity-dependent manner (Dityatev *et al.*, 2000). In co-cultures of NCAM-expressing and NCAM-deficient hippocampal neurons, synapse formation occurs preferentially on NCAM-expressing neurons. However, when glutamatergic transmission is blocked this preference is abolished (Dityatev *et al.*, 2000). For this process the interaction of PSA-NCAM with heparan sulfate proteolgycans and signaling of FGF-receptors are necessary (Dityatev *et al.*, 2004). The ability of NCAM to remodel synaptic strength suggests a function of NCAM in regulating synaptic plasticity. Evidences supporting this hypothesis are the increased expression of NCAM in synaptic spines after long-term potentiation (LTP) (Schuster *et al.*, 1998) and impaired LTP and LTD as well as deficits in spatial learning in NCAM-deficient mice (Bukalo *et al.*, 2004; Cremer *et al.*, 1994; Muller *et al.*, 1996). PSA added to NCAM has been shown to be required for activity-induced synaptic plasticity as its removal abolished induction of LTP and LTD completely (Muller *et al.*, 1996). The importance of PSA-NCAM in synapse remodeling during development and in the mature brain is supported by the fact that hippocampi of schizophrenic brains have reduced levels of PSA-NCAM, possibly causing altered plasticity and connectivity (Barbeau *et al.*, 1995).

SynCAMs:

In contrast to other IgSF-CAM members, which were first characterized as cell adhesion molecules and only later localized to the synapse, the SynCAMs were first identified as synaptic adhesion molecules, as indicated by their name (Biederer *et al.*, 2002). The gene family of synaptic cell adhesion molecules (SynCAMs) comprises four members, SynCAM1-4, in mammals, and three members, SynCAM1-3, in chicken (Biederer *et al.*, 2002; Biederer, 2006; Niederkofler *et al.*, 2010). They are encoded by the cell adhesion molecule 1 to 4 (CADM1-4) genes and consist of three extracellular Ig-like domains, a single transmembrane domain and a short cytoplasmic tail (Biederer *et al.*, 2002; Biederer, 2006). SynCAMs are present throughout the vertebrate genome and are evolutionary strongly conserved

(Biederer, 2006). The cytosolic tail is the most conserved region between family members and between different species (Biederer, 2006). It interacts with intracellular proteins containing a FERM (4.1/ezrin/radixin/moesin)-domain, such as members of the 4.1 protein family or Farp1, and with proteins with a PDZ type II-binding domain, such as the membrane associated guanylate kinase (MAGUK) family members CASK, Pals2 and Dlg3 (Biederer *et al.*, 2002; Cheadle & Biederer, 2012; Hoy *et al.*, 2009; Kakunaga *et al.*, 2005; Shingai *et al.*, 2003; Yageta *et al.*, 2002; Zhou *et al.*, 2005). Like other IgSF-CAMs SynCAMs interact via their extracellular Ig-like domains in a Ca^{2+}-independent manner both homo- and heterophilically (Fogel *et al.*, 2007; Maurel *et al.*, 2007; Niederkofler *et al.*, 2010; Spiegel *et al.*, 2007; Thomas *et al.*, 2008). The strength of SynCAM trans-adhesion is modified by N- and O-glycosylation of the first Ig-domain as it has been shown for SynCAM1 and SynCAM2 (Biederer, 2006; Fogel *et al.*, 2010). Like NCAM, SynCAMs are modified by sialyl transferases (Galuska *et al.*, 2010).

Function of SynCAMs at the synapse

Since their discovery in the synapse, the role of SynCAMs in synaptogenesis has been extensively studied. Evidence that SynCAMs contribute to synapse formation came from heterologous co-culture assays showing that overexpression of SynCAM1 in non-neuronal cells induced functional presynaptic terminal differentiation in co-cultured hippocampal neurons (Biederer *et al.*, 2002; Sara *et al.*, 2005). The induced synapses were functional as they showed spontaneous electrical activity after co-expression of SynCAM1 with glutamate receptors (Biederer *et al.*, 2002; Sara *et al.*, 2005). Overexpression of SynCAM1 in hippocampal neurons increased the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Biederer *et al.*, 2002; Sara *et al.*, 2005). In line with these findings are the complementary effects in transgenic mice overexpressing SynCAM1 and mice lacking SynCAM1, showing an increase and decrease in excitatory synapse number and mEPSC frequency, respectively, without affecting inhibitory synapses (Robbins *et al.*, 2010). Together, this shows that SynCAM1 contributes to excitatory synapse number and function in hippocampal neurons. Only recently a novel role of SynCAM1 in the structural organization and function of ribbon synapses in the mouse retina has been discovered (Ribic *et al.*, 2013). SynCAM1 knockout mice showed a changed ultrastructure and molecular

composition of synaptic ribbon terminals of rod photoreceptors in the outer plexiform layer. Loss of SynCAM1 altered the signal transduction of the rod photoreceptor pathway. Thus, SynCAM1 not only plays a role in classical synapses in the hippocampus but also in ribbon synapses of photoreceptors in the retina. The second family member, SynCAM2, has attracted its attention because of its strong heterophilic interaction with SynCAM1 in synaptosomal membranes (Fogel *et al.*, 2007). SynCAM1 and SynCAM2 assemble into a trans-synaptic adhesive complex thereby organizing functional synapses and promoting excitatory synaptic number and transmission (Fogel *et al.*, 2007).

Prior to the trans-synaptic adhesion, SynCAM1 laterally assembles and clusters into multimeric complexes (Fogel *et al.*, 2011). This clustering is required for recruitment of intracellular effector molecules, such as Farp1 (Cheadle & Biederer, 2012) (Fig. 1). The SynCAM1-Farp1 complex triggers anterograde and retrograde signals inducing polymerization of actin in spines and organizing presynaptic active zones (Cheadle & Biederer, 2012). Another intracellular binding partner of SynCAM1 is the MAGUK family member CASK (Biederer *et al.*, 2002). CASK interacts with components of the presynaptic terminal, including SynCAM1 and neurexins, the synaptic vesicle exocytosis machinery molecules Mint1 and Veli, voltage-gated Ca^{2+}-channels and protein 4.1, which links CASK and the vesicle release machinery to the actin cytoskeleton (Biederer *et al.*, 2002; Butz *et al.*, 1998; Cohen *et al.*, 1998a; Dean *et al.*, 2003; Hata *et al.*, 1996; Hoover & Bryant, 2000; Samuels *et al.*, 2007) (Fig. 1). CASK is also present in the postsynapse interacting with SynCAMs and mediating trafficking of NMDARs to synaptic membrane surface (Biederer *et al.*, 2002; Jeyifous *et al.*, 2009). Hence, CASK and Farp1 together with SynCAMs and neurexins/neuroligins contribute to synaptic signaling in pre- and postsynaptic terminals (Fig. 1). Besides the SynCAM1-Farp1 and SynCAM1-CASK complexes, members of the protein 4.1 family have been identified as postsynaptic effector molecules of SynCAM1 (Hoy *et al.*, 2009) (Fig. 1). SynCAM1 interacts with protein 4.1B/Dal1 and 4.1N through its FERM-binding domain leading to the differential recruitment of NMDAR and AMPAR, respectively (Hoy *et al.*, 2009). Hence, SynCAM1 directly affects excitatory postsynaptic differentiation and stabilization via these effector proteins. Additionally, SynCAM3 interacting with protein 4.1N mediates the recruitment of protein 4.1N to the plasma membrane (Zhou *et al.*, 2005). Association of SynCAM3 with protein 4.1N suggests that SynCAM3 might be involved in synaptic function by recruiting AMPARs via protein 4.1N, as it has been shown for SynCAM1

(Hoy *et al.*, 2009; Zhou *et al.*, 2005). In addition to SynCAM1 expression at excitatory synapses, SynCAM1 has been found to be localized to inhibitory synapses between Purkinje cells and parallel fibers in the cerebellum where it forms a ternary complex with multiple PDZ domain protein 1 (Mupp1) and $GABA_B$ receptor in the postsynapse (Fujita *et al.*, 2012a).

In addition to SynCAM1's function in excitatory synaptogenesis, it is required later in development for the maintenance of excitatory synapses after the peak of synaptogenesis and for the regulation of synaptic plasticity (Lyckman *et al.*, 2008; Robbins *et al.*, 2010; Zelano *et al.*, 2009). Overexpression of SynCAM1 abrogates loss of synapses during long-term depression (LTD) whereas loss of SynCAM1 increases LTD (Robbins *et al.*, 2010). Moreover the SynCAM-dependent decrease or increase in LTD has a direct effect on cognitive functions affecting spatial learning and memory in mice. Hence, different to other IgSF-CAMs stabilizing LTP and LTD (Bukalo *et al.*, 2004; Kim *et al.*, 2008b; Murai *et al.*, 2002), SynCAM1 prevents LTD without affecting LTP (Robbins *et al.*, 2010). A role of SynCAM1 in synaptic plasticity has been further demonstrated by the increased expression of SynCAM1 in the visual cortex after monocular deprivation (Lyckman *et al.*, 2008). In the mature nervous system SynCAM1 has been implicated in regeneration of motor axons after nerve axotomy, where downregulation and upregulation of SynCAM1 expression correlated with loss and return of synapses on motoneurons, respectively (Zelano *et al.*, 2009; Zelano *et al.*, 2007).

The two other members of the SynCAM family, SynCAM3 and SynCAM4, are also prominently expressed in the brain and enriched in the synaptic plasma membrane during the period of synaptogenesis (Fogel *et al.*, 2007). SynCAM3 has not been found at synaptic contacts but rather at non-junctional sites where axon terminals and astrocyte processes contact to surround pre- and postsynapses (Kakunaga *et al.*, 2005). This suggests that SynCAM3 is involved in the formation of astrocytic processes, which is important for the segregation of synapses from each other. However, the ability of SynCAM3 to interact with MAGUK proteins, such as CASK, and protein 4.1N indicates that SynCAM3 might be involved in the organization and function of presynaptic specializations and the recruitment of receptors to the postsynaptic membrane (Butz *et al.*, 1998; Kakunaga *et al.*, 2005; Samuels *et al.*, 2007; Zhou *et al.*, 2005). Hence, although their role in synapse formation and function

remains to be investigated, SynCAM3 and, due to the strong heterophilic interaction, also SynCAM4 are promising candidates (Fogel *et al.*, 2007; Thomas *et al.*, 2008).

Contribution of SynCAMs to myelination of PNS and CNS axons

In addition to their synaptic functions, SynCAMs have been implicated in myelination in the peripheral (PNS) as well as the central nervous system (CNS). Myelination by Schwann cells in the peripheral nervous system is induced by the interaction between axonal SynCAM1 and SynCAM3 with Schwann cell SynCAM4 (Maurel *et al.*, 2007; Spiegel *et al.*, 2007). SynCAMs localize at the axo-glial interface along the internode of myelinated fibers but are excluded from the nodes and paranodes. In the central nervous system SynCAM2 and SynCAM3 have been implicated in myelination, although reports on their detailed expression in axons versus glia differ (Kakunaga *et al.*, 2005; Park *et al.*, 2008; Pellissier *et al.*, 2007).

Involvement of SynCAMs in axonal pathfinding

In line with their membership in the IgSF-CAM superfamily, SynCAMs have been shown to affect guidance of commissural axons (Niederkofler *et al.*, 2010). The dI1 subpopulation of commissural neurons extends axons ventrally towards the floor plate. Axons enter the floor plate to cross the midline due to positive signals derived from the interaction between growth cone CNTN1/TAG1/axonin-1 and floor-plate NrCAM (Stoeckli & Landmesser, 1995; Stoeckli *et al.*, 1997). A switch from attraction to repulsion expels the growth cones out of the floor plate. This switch in surface receptor expression is mediated by RabGDI, which triggers the insertion of the IgSF-CAM Robo1 (Philipp *et al.*, 2012). This in turn, allows growth cones to detect Slits, the negative guidance cues associated with the midline area. Once commissural axons exit the floor plate, they turn rostrally along the longitudinal axis directed by two opposing morphogen gradients, an attractive Wnt and a repulsive Shh gradient (Stoeckli, 2006). SynCAM1 and SynCAM2 have been found to be required for post-crossing commissural axon guidance at the contralateral border of the FP and their turning response in the embryonic chicken spinal cord (Niederkofler *et al.*, 2010). Perturbation of SynCAM1 and SynCAM2 in commissural axons and of SynCAM2 expression in the floor plate resulted in aberrant projections of commissural axons at the midline. Instead of making a rostral turn,

axons stalled at the contralateral border of the floor plate. These results extend the current knowledge of SynCAM functions and reveal their involvement in steps of neural circuit formation prior to synaptogenesis and myelination.

Disease-associated mutations in SynCAM1 gene

As discussed in the previous chapters, mutations in synaptic cell adhesion molecules like neuroligins/neurexins, LRRTMs, N-cadherins, NgCAM/NrCAM, contactins and NCAM have been implicated in autism and other cognitive diseases, such as intellectual disability and schizophrenia. Also in the gene encoding SynCAM1, two missense mutations, H246N and Y251S, have been found in patients with ASD (Zhiling *et al.*, 2008). This linkage has also been validated in SynCAM1 knockout mice, which exhibit deficits in social and emotional behavior as well as impaired ultrasonic vocalization (Fujita *et al.*, 2012b; Takayanagi *et al.*, 2010). The two missense mutations are located in the third Ig-like domain of SynCAM1, a region important for mediating adhesion (Fujita *et al.*, 2010; Zhiling *et al.*, 2008). The mutations induce a conformational change of the protein probably causing defective glycosylation. Misfolding and defective glycosylation could lead to impaired trafficking of SynCAM1 to the cell surface, increased susceptibility for degradation and intracellular accumulation, mainly in the endoplasmic reticulum (ER) (Zhiling *et al.*, 2008). Intracellular accumulation of mutated SynCAM1 causes ER stress by upregulation of CHOP, a regulator of membrane trafficking thus controlling synaptic function (Fujita *et al.*, 2010). Autism-linked gain-of-function mutation in the NLG3 gene has also been associated with intracellular retention and ER stress (Comoletti *et al.*, 2004; Fujita *et al.*, 2010; Tabuchi *et al.*, 2007). Thus, ER stress induced by misfolding and disrupted trafficking of these mutant proteins might add another cause underlying the pathology of ASD (Fujita *et al.*, 2010; Momoi *et al.*, 2010).

Dysfunction of synaptic cell adhesion molecules may contribute to the pathogenesis of neurodevelopmental disorders by affecting neural circuit formation during multiple steps

Synaptic cell adhesion molecules, including neuroligins/neurexins, LRRTMs, N-cadherins, NgCAM/NrCAM, contactins, NCAM and the SynCAMs, do by far more than just mediating adhesion between synapses. They are important for synapse function, maintenance and plasticity. Although each class of adhesion molecule seems to play a specific role in synaptogenesis they all have in common that they affect synaptic function. In addition, many of them interact with each other or share common intracellular binding partners (Fig. 1). Mutations in these genes were therefore thought to contribute to neurodevelopmental diseases, such as ASD, ID and/or schizophrenia, by affecting synaptic function and plasticity. However, it may well be that the contribution of IgSF-CAMs, both the 'classical' groups, such as NCAM, the L1 subgroup, and the contactin subgroup, as well as the more recently described SynCAMs to neural circuit formation exceeds their role in synaptogenesis and synaptic plasticity. The 'classical' IgSF-CAMs have been well characterized as axon guidance cues (Stoeckli, 2004). The contribution of the SynCAMs to axon guidance is a more recent finding (Niederkofler *et al.*, 2010). Such a function has not yet been analyzed for LRRTMs, neuroligins, and neurexins. However, it would not be too surprising to find this feature conserved among so-called synaptic cell adhesion molecules. An additional role in early aspects of neural circuit formation would parallel findings for the morphogens. These molecules are involved classically in cell fate determination during early development (Ashe & Briscoe, 2006; Briscoe & Ericson, 2001). Later, they are involved in axon guidance (Stoeckli, 2006; Zou & Lyuksyutova, 2007), and finally the affect synaptogenesis and synaptic plasticity (Salinas & Zou, 2008).

Because some synaptic cell adhesion molecules are involved in many different steps during the development of neural circuits, their association with neurodevelopmental diseases could underlie more than just impaired synaptic function and plasticity. Their roles in axon elongation and pathfinding could contribute to autism and other cognitive diseases. The importance of synaptic cell adhesion molecules before the onset of synapse formation could also help explain why neurodevelopmental disorders have both overlapping but also distinct features. Obviously they all share a deficit in synaptic function and plasticity. However,

based on the nature of the affected genes, distinct circuits may have been affected already during wiring, and, thus, give rise to a different variant of ASD and/or intellectual disability.

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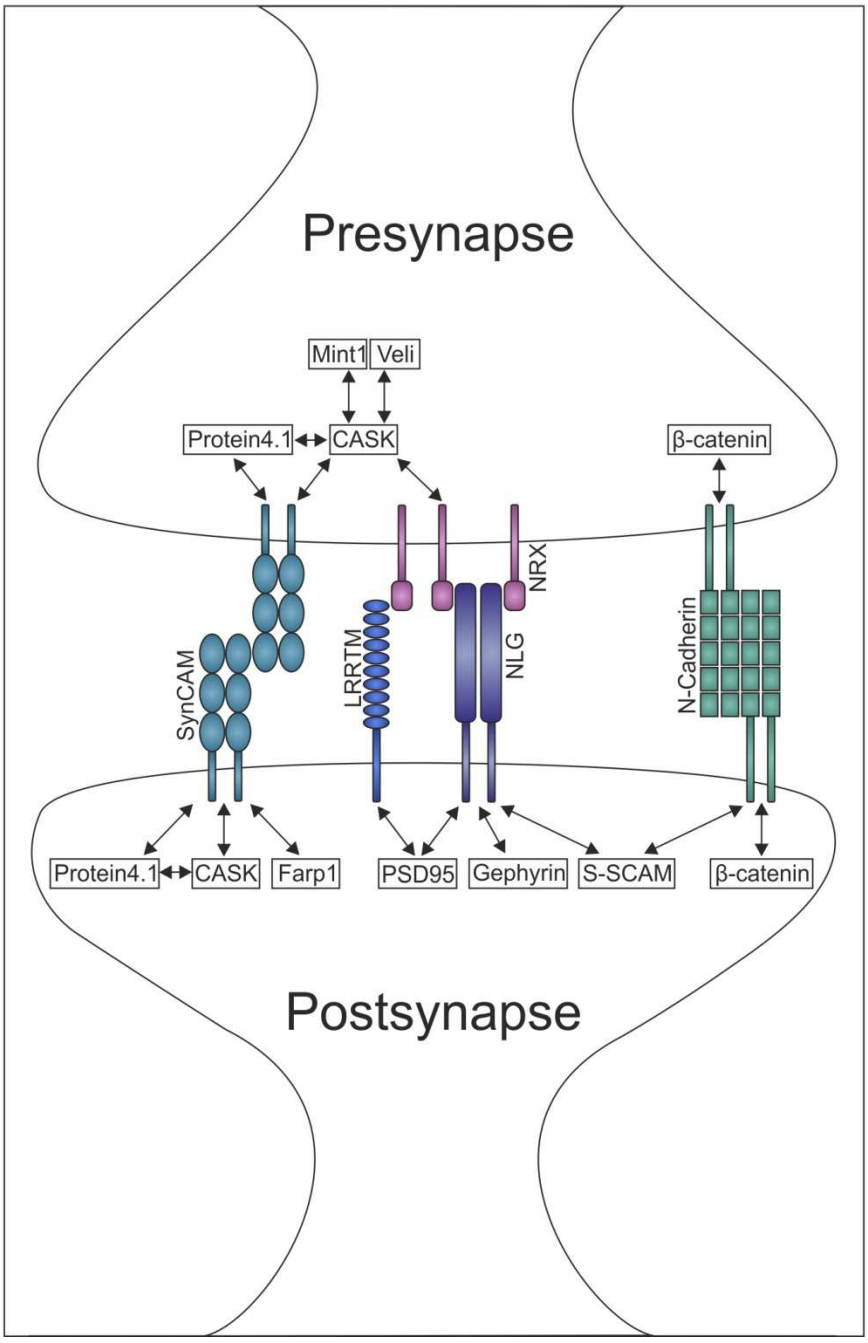
Figure 1

Synaptic cell adhesion molecules form trans-synaptic complexes to link pre- and postsynaptic elements. Synaptic cell adhesion is mediated by SynCAMs, neuroligins and neurexins, LRRTMs, and N-cadherins. SynCAMs engage in homophilic as well as heterophilic interactions, whereas N-cadherins only mediate homophilic adhesion. Neurexins can interact with neuroligins and LRRTMs. To organize pre- and postsynapse, they recruit various scaffold proteins. In the postsynapse, SynCAMs bind to the scaffold molecule CASK, actin-binding proteins of the 4.1 family, and the guanine nucleotide exchange factor Farp1. Neuroligins and LRRTMs both recruit PSD95 in excitatory synapses. In inhibitory synapses, neuroligins are connected to gephyrin scaffolds. N-cadherin binds to β-catenin and clusters neuroligin via the scaffolding molecule S-SCAM. In the presynapse both SynCAMs and neurexins bind to CASK. CASK in turn forms a complex with Mint1 and Veli.

Figure 1. Synaptic cell adhesion molecules form trans-synaptic complexes to link pre- and postsynaptic elements



Review: SynCAMs extend their functions beyond the synapse

SynCAMs extend their functions beyond the synapse

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Abstract

Synaptic cell adhesion molecules have been identified due to their potential to trigger synaptogenesis in vitro even when expressed in non-neuronal cell lines. In addition to the SynCAMs, other structurally unrelated families of synaptic cell adhesion molecules have been identified: Neurexins and neuroligins, as well as the LRRTMs, the leucine-rich repeat transmembrane neuronal protein family. Although in vivo the absence of individual synaptic cell adhesion molecules does not necessarily reduce the number of synapses, it does affect the function of synapses. Not surprisingly, mutations in synaptic cell adhesion molecules have been identified in patients suffering from neurodevelopmental disorders, such as autism spectrum disorders, intellectual disability, or schizophrenia. In line with the major function of these genes at the synapse their role in the pathogenesis of neurodevelopmental diseases has been attributed to synaptogenesis, synapse maintenance, and synaptic plasticity. However, SynCAMs have been implicated also in axon guidance, that is, an earlier step in neural circuit formation. These findings suggest that SynCAMs, and maybe other families of synaptic cell adhesion molecules as well, could contribute to the pathogenesis of neurodevelopmental disorders at multiple steps of neural circuit formation, and thus, shape the distinct symptoms associated with different disease variants or distinct neurodevelopmental disorders in addition to their effect on synaptic function.

Introduction

Neural circuits are the building blocks of the nervous system. Changes in the efficacy of information transfer by changing synaptic strength are the basis of cognitive processes. Thus, it is not surprising that synaptogenesis and synaptic plasticity attract a lot of attention. Deficits in synaptic plasticity cause neural diseases throughout the spectrum from neurodevelopmental to neurodegenerative diseases. Therefore, the identification of the underlying molecular mechanisms has been a major goal of neuroscience research during the last decade.

Conceptually, it makes a lot of sense that cell adhesion molecules specifically located at synaptic contact sites trigger and organize synaptogenesis. First of all, it is important to have some glue keeping pre- and postsynaptic elements tightly bound together. Furthermore, you need to have a link to intracellular scaffold molecules which in turn could recruit and anchor the synaptic vesicle release machinery. Traditionally, cadherins have been implicated in synaptic adhesion and synapse organization (Takeichi & Abe, 2005; Missler *et al.*, 2012). However, it has become clear that they would not be sufficient to explain synapse formation and function. A new era in synaptic cell adhesion has started with the demonstration that neuroligins expressed in non-neuronal cell lines can induce the formation of presynaptic specializations (Scheiffele *et al.*, 2000). This assay was adopted as a defining feature for synaptic cell adhesion molecules to distinguish them from more ubiquitously expressed cell adhesion molecules.

The synaptic cell adhesion molecules SynCAMs were identified based on structural criteria and their function as homophilic cell adhesion molecules (Biederer *et al.*, 2002). Because of their localization at the synapse and their potential to induce synaptic features when expressed in cell lines, they were called Synaptic Cell Adhesion Molecules. More recently, the in vitro synaptogenesis assay has been used to screen for additional candidate synaptogenic molecules, resulting in the characterization of the leucine-rich repeat transmembrane neuronal protein family (LRRTMs; Linhoff *et al.*, 2009).

Synaptic cell adhesion molecules have multiple functions at the synapse. They trigger synaptogenesis, contribute to synaptic organization, specification and maturation, but are also important for maintenance and plasticity of synapses (Gerrow & El-Husseini, 2006; Dalva *et al.*, 2007; Bukalo & Dityatev, 2012). Based on their central role for synaptic

structure and function, it does not come as a surprise that mutations in synaptic cell adhesion molecules have been identified as cause or contributor to neurodevelopmental diseases, such as intellectual disability or autism spectrum disorders (Zoghbi & Bear, 2012; Melom & Littleton, 2011). However, at least one family of synaptic cell adhesion molecules, the SynCAMs, have functions that go beyond the synapse, as they have been implicated in myelination and axon guidance. Therefore, the synaptic cell adhesion molecules may specify the symptoms and the type of neurodevelopmental disorder by affecting some neural circuits more specifically than others. In this review, we summarize current knowledge on the role of one family of synaptic cell adhesion molecules, the SynCAMs, in multiple steps of neural circuit formation.

SynCAMs form a subgroup of the immunoglobulin-superfamily of cell adhesion molecules

In contrast to other subgroups of the immunoglobulin-superfamily of cell adhesion molecules (IgSF-CAMs), SynCAMs were first identified as synaptic cell adhesion molecules, as indicated by their name (Biederer *et al.*, 2002). For all other subgroups, a role in axon fasciculation, axon growth, and axonal pathfinding was characterized first, and their localization to the synapse has only been confirmed later (Bukalo & Dityatev, 2012; Stoeckli, 2004; Rougon & Hobert, 2003). SynCAMs are encoded by the cell adhesion molecule 1 to 4 (CADM1-4) genes (Thomas *et al.*, 2008). These genes had been identified independently in different contexts and, therefore, have multiple names (Table 1).

SynCAMs are present throughout the vertebrate genome and are evolutionary highly conserved (Biederer, 2006). The SynCAM/CADM family comprises four members in mammals, SynCAM1-4, and three members in chicken, SynCAM1-3 (Biederer, 2006; Biederer *et al.*, 2002; Niederkofler *et al.*, 2010). Their cytosolic tail is the most conserved region both between the different family members but also between species. It interacts with protein 4.1 and PDZ type II-domain proteins (Hoover & Bryant, 2000; Hung & Sheng, 2002). Via the protein 4.1 family members, 4.1B/Dal1 and 4.1N, SynCAMs are linked to the actin cytoskeleton (Hoy *et al.*, 2009; Yageta *et al.*, 2002; Zhou *et al.*, 2005). Via PDZ-scaffold proteins SynCAMs are involved in the organization of protein complexes at the synapse. SynCAMs specifically interact with PDZ-domain proteins of the membrane associated guanylate kinase (MAGUK) family (Montgomery *et al.*, 2004), such as CASK

(Calcium/calmodulin-dependent serine protein kinase), Pals2, and Dlg3 (Biederer *et al.*, 2002; Kakunaga *et al.*, 2005; Shingai *et al.*, 2003). Via their extracellular Ig-like domains SynCAMs mediate Ca^{2+}-independent homo- and heterophilic adhesion. SynCAM1, 2 and 3 but not SynCAM4 engage in homophilic complexes (Biederer *et al.*, 2002; Fogel *et al.*, 2007; Kakunaga *et al.*, 2005; Shingai *et al.*, 2003). Heterophilic interaction is generally stronger than homophilic adhesion and occurs mainly between SynCAM1 and -2, between SynCAM3 and -4 and between SynCAM2 and -4 (Fogel *et al.*, 2007; Maurel *et al.*, 2007; Niederkofler *et al.*, 2010; Thomas *et al.*, 2008). In addition, SynCAMs also interact with Nectins, another structurally related IgSF-CAM subgroup (Kakunaga *et al.*, 2005; Shingai *et al.*, 2003).

Like other IgSF-CAMs SynCAMs can interact in cis and in trans. SynCAM1 was shown to form cis-oligomers prior to homo- or heterophilic trans-interaction (Fogel *et al.*, 2011). For cis-assembly the Ig-like domains 2 and 3 are required, whereas the first Ig-domain was shown to be required for trans-interaction (Fogel *et al.*, 2007; Fogel *et al.*, 2011). Blocking lateral assembly of SynCAM1 or removal of the first Ig domain results in a reduced ability to bind to SynCAM2. The adhesive strength of SynCAM trans-interactions is modified by N-glycosylation of the first Ig-domain of SynCAM1 and SynCAM2 (Fogel *et al.*, 2010). N-glycosylation in the first Ig-domain of SynCAM1 promotes homo- and heterophilic adhesion to SynCAM2. In contrast, the same modification in SynCAM2 reduces its adhesive interactions with SynCAM1 and SynCAM2 (Fogel *et al.*, 2010). A subpopulation of SynCAM1 isoforms, generated by alternative splicing, was shown to be modified by polysialylation of N-glycans (Fogel *et al.*, 2007; Galuska *et al.*, 2010). In addition to N-glycosylation, potential sites for O-glycosylation have been identified in SynCAM1 and SynCAM2, but not SynCAM3 and SynCAM4 (Biederer, 2006).

SynCAMs contribute to myelination

In line with a role in cell-cell junction organization in non-neuronal cells (Takai *et al.*, 2008; see Table 1), SynCAMs contribute to myelination both, in the peripheral (PNS) and the central nervous system (CNS). Peripheral axons express SynCAM1 and SynCAM3, whereas Schwann cells express SynCAM1 and high levels of SynCAM4 (Maurel *et al.*, 2007; Spiegel *et al.*, 2007). SynCAMs localize to the axon-glia interface along the internode of myelinated fibers but are excluded from the nodes. The role of SynCAM1 in myelination is not fully understood mainly due to a missing interaction partner on Schwann cells (Maurel *et al.*, 2007). In contrast, much more is known about the function of SynCAM3 and SynCAM4 in PNS myelination. SynCAM3 on axons specifically binds to Schwann cell-expressed SynCAM4, thereby mediating adhesion between axons and Schwann cells (Maurel *et al.*, 2007; Spiegel *et al.*, 2007). Knockdown of SynCAM4 or disruption of the SynCAM3-SynCAM4 interaction resulted in inhibition of myelination in co-cultures of DRG neurons and Schwann cells, although the alignment of Schwann cells with axons was not perturbed. This finding suggested that SynCAMs were not required for initial axon-glia contact but maybe for proper Schwann cell polarity and subsequent myelination (Maurel *et al.*, 2007; Spiegel *et al.*, 2007). Recently, these findings have been questioned because disruption of SynCAM4 in mice did not affect myelination in the PNS and CNS, suggesting that SynCAM4 might not be required for myelination in vivo (Zhu *et al.*, 2013). A possible explanation for the discrepancy between in vitro and in vivo studies could be compensatory mechanisms that rescue the loss of SynCAM4 in knockout mice but not in co-cultures in vitro.

In the developing central nervous system, a delay in myelination in the optic nerve and in the spinal cord was found in SynCAM3-knockout mice (Park *et al.*, 2008). Park and colleagues found SynCAM3 to be exclusively expressed in CNS neurons in contrast to results published earlier by Kakunaga and colleagues, who found SynCAM3 both in axons and glia cells (Kakunaga *et al.*, 2005). At this point it is not clear how this discrepancy can be explained. While Park and colleagues used in situ hybridization to exclude SynCAM3 expression from the white matter, Kakunaga and colleagues used an antibody to demonstrate SynCAM3 expression in astrocytes surrounding axon terminals. In addition to SynCAM3, SynCAM2 was discovered in myelinated axons in the CNS (Pellissier *et al.*, 2007). SynCAM2 was shown to accumulate at contact sites between axons and oligodendrocytes. So far, the binding partner on oligodendrocytes has not been identified.

SynCAMs at the synapse

SynCAMs are sufficient to induce synapses

As mentioned above, SynCAMs fulfill the gold standard for synaptic cell adhesion molecules: their expression in a non-neuronal cell line is sufficient to trigger the formation of presynaptic specializations in co-cultured hippocampal neurons (Biederer *et al.*, 2002; Sara *et al.*, 2005). Co-expression of SynCAM1 with glutamate receptors in non-neuronal cells resulted in spontaneous electrical activity when co-cultured with neurons. Overexpression of SynCAM1 in hippocampal neurons increased the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) at excitatory synapses (Biederer *et al.*, 2002; Sara *et al.*, 2005). These in vitro effects on synaptogenesis were confirmed in vivo, as transgenic mice overexpressing SynCAM1 showed an increase, whereas mice lacking SynCAM1 showed a decrease in excitatory synapse number and mEPSC frequency, without affecting inhibitory synapses (Robbins *et al.*, 2010). Taken together, these findings confirm a contribution of SynCAM1 to excitatory synapse number and function in hippocampal neurons.

Recently, a role of SynCAM1 in the structural organization and function of ribbon synapses in the mouse retina has been discovered (Ribic *et al.*, 2013). SynCAM1 knockout mice showed an aberrant molecular composition of synaptic ribbon terminals of rod photoreceptors in the outer plexiform layer. As a consequence both ultrastructure of ribbon synapses and signal transduction in the rod photoreceptor pathway was affected.

SynCAMs contribute to the maintenance of synapses

SynCAM1's function is not restricted to synaptogenesis, as it is also required for the maintenance of excitatory synapses, in agreement with its persistent expression after the peak of synaptogenesis and in the adult brain, where it was found to regulate synaptic plasticity (Robbins *et al.*, 2010; Thomas *et al.*, 2008). Overexpression of SynCAM1 abrogates loss of synapses during long-term depression (LTD), whereas loss of SynCAM1 increases LTD (Robbins *et al.*, 2010). Moreover the SynCAM-dependent decrease or increase of LTD has a direct effect on cognitive functions affecting spatial learning and memory in mice. The involvement of SynCAM1 in synaptic plasticity has been demonstrated by the increased expression of SynCAM1 in the visual cortex after monocular deprivation (Lyckman *et al.*,

2008). The importance of SynCAMs for synapse maintenance was also shown in the spinal cord, where SynCAM1 levels decreased prior to synapse loss on motoneurons after sciatic nerve transsection (Zelano *et al.*, 2009; Zelano *et al.*, 2007). During regeneration of motor axons, SynCAM1 expression recovered.

SynCAMs organize pre- and postsynaptic sites via intracellular binding partners

Prior to synapse formation, contact between axons and dendrites results in a rapid clustering of SynCAM1 (Fogel *et al.*, 2011; Stagi *et al.*, 2010). Subsequently, the cis-assembly of SynCAM1 promotes adhesive trans-binding to SynCAMs, most probably SynCAM2, across the synaptic cleft which in turn leads to the induction of functional presynaptic specializations (Fogel *et al.*, 2007; Fogel *et al.*, 2011). SynCAM1 cis-assembly contributes to the organization of the synaptic structure by restricting the size of the pre- and postsynaptic specializations (Fogel *et al.*, 2011). How does this trans-synaptic SynCAM interaction transmit an intracellular signal which finally leads to the formation of functional synapses? The intracellular protein Farp1 has been suggested to act downstream of SynCAM1 in excitatory postsynapses, to control synapse density, and to promote excitatory synapse formation by affecting filopodial dynamics (Cheadle & Biederer, 2012). Farp1 is a guanine nucleotide exchange factor (GEF) which is prominently expressed in dendritic spines. It interacts via its FERM domain with the cytoplasmic tail of SynCAM1 (Fig. 1). Via its DH (Dbl oncogene homology) and PH (pleckstrin homology) domains Farp1 activates Rac1 and, thereby, promotes actin polymerization in spines, which in turn contributes to spine dynamics.

The SynCAM1/Farp1 interaction triggers a retrograde signal across the synaptic cleft which modulates the composition of the presynaptic active zone. This is in line with SynCAM1's function in controlling the organization of presynaptic structures (Fogel *et al.*, 2011; Robbins *et al.*, 2010). Importantly, for retrograde trans-synaptic signaling and for postsynaptic Rac1 activation, Farp1 requires the interaction with clustered SynCAM1, highlighting the importance of SynCAM1 cis-assembly for synaptic signaling (Cheadle & Biederer, 2012; Fogel *et al.*, 2011).

In addition to Farp1, the MAGUK family member CASK binds to the intracellular domain of SynCAM1 and SynCAM3 (Biederer *et al.*, 2002; Kakunaga *et al.*, 2005) (Fig. 1). CASK has been

originally identified as intracellular binding partner of neurexin (Hata *et al.*, 1996). Phosphorylation of CASK by the serine/threonine kinase Cdk5 localizes CASK to synaptic membranes where it is capable to interact with components of the presynaptic terminal and importantly with neurexins, the synaptic vesicle exocytosis machinery molecules Mint1 and Veli, voltage-gated Ca^{2+}-channels, and protein 4.1, which links CASK and the presynaptic vesicle release machinery to the actin cytoskeleton (Butz *et al.*, 1998; Cohen *et al.*, 1998; Dean *et al.*, 2003; Hoover & Bryant, 2000; Samuels *et al.*, 2007) (Fig. 1). SynCAM1 has also been shown to directly interact with Mint1 (Biederer *et al.*, 2002). Taken together, SynCAM1's interactions with Farp1 and CASK provide a model how SynCAMs can impact the organization of presynaptic terminals.

CASK is also present in the postsynapse, where its interaction with SynCAMs could contribute to the recruitment of NMDA-receptors (NMDARs) to the postsynaptic membrane (Biederer *et al.*, 2002; Jeyifous *et al.*, 2009). Hence, CASK and Mint are downstream of both SynCAMs and neurexins/neuroligins and link their contributions to pre- and postsynaptic organization and function (Fig. 1). A possible model for the induction of excitatory synapses by SynCAMs would include a SynCAM1 cluster interacting with Farp1 in the postsynapse. Postsynaptic SynCAM1 would interact trans-synaptically with SynCAM2 in the presynaptic membrane. As the SynCAM1-SynCAM2 heterophilic interaction is stronger than the SynCAM1 homophilic trans-interaction, SynCAM2 is probably the preferred binding partner for trans-synaptic signaling (Fogel *et al.*, 2007). In the presynapse, SynCAM2 would recruit CASK and thereby affect presynaptic function. Although a direct interaction between SynCAM2 and CASK has not been demonstrated yet, it is very likely as the cytoplasmic domains of SynCAM family members are highly conserved (Biederer, 2006). As SynCAM2 does not interact with Farp1 (Cheadle & Biederer, 2012), SynCAM2 localized to the postsynapse could modulate trans-synaptic signaling of SynCAM1.

Besides the SynCAM1-Farp1 and SynCAM1-CASK complexes, proteins of the 4.1 family have been identified as postsynaptic effector molecules of SynCAM1 (Hoy *et al.*, 2009) (Fig. 1). Association of SynCAM1 with protein 4.1B, also named Dal1, has previously been reported in the formation of stable cell adhesion, as loss of this interaction contributed to the formation of lung tumors (Yageta *et al.*, 2002). SynCAM1 interacts with protein 4.1B and 4.1N through its FERM binding domain, and, thus, indirectly contributes to the differential recruitment of

NMDAR and AMPA receptors (AMPARs), respectively, to postsynaptic sites (Hoy *et al.*, 2009). Hence, SynCAM1 affects excitatory postsynaptic differentiation and stabilization via these linker proteins. Additionally, an interaction between SynCAM3 and protein 4.1N has been reported to mediate the recruitment of protein 4.1N to the plasma membrane (Zhou *et al.*, 2005). Thus, SynCAM3 could cooperate with SynCAM1 in the recruitment of AMPARs via protein 4.1N.

In addition to its expression at excitatory synapses in cortex and hippocampus, SynCAM1 was localized to inhibitory synapses between Purkinje cells and parallel fibers in the cerebellum. There, SynCAM1 not only interacts with CASK, but also binds to Mupp1 (Multiple PDZ domain protein 1), which binds GABA type B receptors in the postsynapse (Fujita *et al.*, 2012a).

Mutations in SynCAM are associated with neurodevelopmental diseases

In line with their function in synaptogenesis, synaptic organization and plasticity, mutations in neuroligins and neurexins have been linked to autism spectrum disorders (ASD) and intellectual disability (Jamain *et al.*, 2003; Yan *et al.*, 2005; Kim *et al.*, 2008; Dean and Dresbach, 2006; Südhof, 2008; Bourgeron, 2009). As SynCAMs cooperate with neurexins and neuroligins at the synapse and as they even share some of their intracellular interaction partners, such as CASK, it is not surprising that SynCAM mutations were also found associated with neurodevelopmental diseases.

Two missense mutations in SynCAM1, H246N and Y251S, were found in patients with ASD (Zhiling *et al.*, 2008). It is impossible to reproduce all the classical autism symptoms, like impaired social interaction and communication, as well as stereotyped behavior in a mouse model. Still, SynCAM1 knockout mice exhibit deficits in some traits linked to ASD, as they were shown to have changes in social and emotional behavior, as well as impaired ultrasonic vocalization (Fujita *et al.*, 2012b; Takayanagi *et al.*, 2010). At the anatomical level, neurons expressing mutant SynCAM1 exhibit aberrant spines and defective synaptic function (Fujita *et al.*, 2010). The two missense mutations are located in the third Ig-like domain of SynCAM1, a region that is important for homo- and heterophilic adhesion. Misfolding and/or defective glycosylation may lead to impaired trafficking of SynCAM1 to the cell surface, due

to increased susceptibility for degradation and intracellular accumulation, mainly in the endoplasmic reticulum (ER) (Zhiling *et al.*, 2008). Intracellular accumulation of mutated SynCAM1 has been shown to cause ER stress by upregulation of CHOP, a regulator of membrane trafficking, and, thus, indirectly affecting synaptic function (Fujita *et al.*, 2010). Autism-linked gain-of-function mutation in the neuroligin3 gene has also been associated with intracellular retention and ER stress (Comoletti *et al.*, 2004; Fujita *et al.*, 2010; Tabuchi *et al.*, 2007). Thus, both loss- and gain-of-function mutations interfere with protein trafficking, intracellular accumulation and ER stress resulting in synaptic dysfunction linked to the pathology of ASD (Fujita *et al.*, 2010; Momoi *et al.*, 2010).

A novel role of SynCAMs in axon guidance

In addition to its functions in synaptogenesis and myelination, which are both late processes in the neural circuit formation, SynCAM2 was identified in a screen for axon guidance cues at the floor plate (Niederkofler *et al.*, 2010). The dI1 subpopulation of commissural neurons is located in the dorsal spinal cord. Their axons extend ventrally towards the floor plate, the ventral midline of the spinal cord, in response to both chemorepulsive and chemoattractive cues (Chédotal, 2011; Nawabi and Castellani, 2011). Commissural axons enter the floor plate to cross the midline due to positive signals derived from the interaction between axonal axonin1/contactin2 and floor-plate NrCAM (Stoeckli & Landmesser, 1995, Stoeckli *et al.*, 1997). The contact between commissural growth cones and the floor plate induces a switch in axonal responsiveness from attraction to repulsion, explaining why axons leave the floor plate on the contralateral side (Philipp *et al.*, 2012). At the floor-plate exit site post-crossing commissural axons turn rostrally along the longitudinal axis in response to morphogen gradients (Zou and Lyuksyutova, 2007; Stoeckli, 2006). A caudal^{high} to rostral^{low} gradient of Shh repels post-crossing axons (Bourikas *et al.*, 2005; Wilson and Stoeckli, 2013), whereas a rostral^{high} to caudal^{low} gradient of Wnts was shown to attract post-crossing axons (Lyuksyutova *et al.*, 2003; Domanitskaya *et al.*, 2010).

In addition to the morphogen gradients, SynCAM1 and SynCAM2 were found to be required for post-crossing commissural axon guidance at the contralateral border of the floor plate in the embryonic chicken spinal cord (Niederkofler *et al.*, 2010). SynCAM1, SynCAM2 and SynCAM3 are expressed in dI1 commissural neurons and SynCAM2 and SynCAM3 are also

expressed by floor-plate cells during the time window of commissural axon pathfinding. The use of in ovo RNAi (Pekarik *et al.*, 2003) to specifically downregulate SynCAMs in neurons or their intermediate target, the floor plate, demonstrated a requirement for SynCAM1 and SynCAM2 in commissural axons and SynCAM2 in the floor plate. Instead of making a rostral turn, axons stalled at the contralateral floor-plate border.

In line with previous studies (Fogel *et al.*, 2007; Thomas *et al.*, 2008), a strong interaction between SynCAM1 and SynCAM2 was demonstrated, suggesting an interaction between axonal SynCAM1 and floor-plate SynCAM2 in post-crossing commissural axon guidance (Niederkofler *et al.*, 2010). However, the homophilic interaction of SynCAM2 is only very weak (Fogel *et al.*, 2007), if present at all (Niederkofler *et al.*, 2010), and, thus, cannot explain the erroneous axonal navigation after knockdown of SynCAM2 in commissural axons. The most parsimonious model explaining the observed phenotypes after knockdown of SynCAM1 and SynCAM2 either in neurons or the floor plate was the formation of heterophilic SynCAM1-SynCAM2 complexes in the commissural growth cone membranes which in turn would interact in trans with SynCAM2 clusters on floor-plate cells. Although the formation of cis-complexes is a well-known feature of SynCAMs, heterophilic oligomers have not been described so far.

Through heterophilic cis-interactions axonal SynCAM2 could modulate the adhesive strength of the SynCAM1-SynCAM2 trans-interaction. A decrease in adhesive strength between commissural axons and floor-plate cells could facilitate the turning response into the longitudinal axis of the spinal cord. Too much adhesion in the absence of axonal SynCAM2 would hence lead to too much stickiness and prevent axons from turning efficiently and from growing rostrally along the anterior-posterior axis. In line with this model, too little adhesion after silencing of axonal SynCAM1 or floor-plate-derived SynCAM2 could result in loss of proper cell-cell contact and as a consequence prevent the perception of guidance information directly or indirectly by interfering with a contact-derived signal changing the receptor expression pattern. Such a change is required to switch the attractive response of the growth cone to the floor plate to a repulsive response. We have shown recently that commissural neurons express RabGDI at the time of growth cone/floor plate contact, resulting in the insertion of Robo1 receptors into the growth cone surface, which in turn leads to the perception of negative floor-plate-associated cues, the Slits (Philipp *et al.*, 2012).

In addition, we have shown that transcription of Hhip, the Shh receptor required for the rostral turn of post-crossing commissural axons, is induced by Shh itself in a Glypican1-dependent manner (Wilson and Stoeckli, 2013). Although, neither the causal requirement of floor plate/growth cone contact nor the actual signal are known, these observations suggest that a contact-derived signal dependent on growth cone/floor plate interaction is required to prepare pre-commissural axons for their pathfinding in their post-crossing phase. Thus, changing axon/floor plate adhesion could interfere with the required changes in guidance receptor expression, and result in the failure of post-crossing axons to move on with the next stage of their journey.

The modulation of cell-cell contacts via changes in cis-interactions of receptors appears to be a general concept in axon guidance. During hindlimb innervation, cis-interactions between Ephs and ephrins were found to be crucial for the fine tuning of responsiveness of axons (Kao & Kania, 2011). EphA and EphB receptors are expressed in lateral and medial motoneurons, respectively, whereas their ligands, the ephrinAs and ephrinBs, are expressed in the ventral and dorsal hindlimb. Co-expression of high levels of ephrins in motor axons was shown to attenuate the corresponding Eph receptor and thereby fine-tune the accurate motor trajectory. Similarly, semaphorin3a was found to modulate neuropilin1-dependent sensitivity of motor axons to limb semaphorin3s (Moret *et al.*, 2007). PlexinD1 cis-interactions with neuropilin1 switched the axonal response to semaphorin3E (Chauvet *et al.*, 2007). A cis-interaction between semaphorin6A and plexinA4 was shown to attenuate the repulsive activity of semaphorin6A for sensory axons (Haklai-Topper *et al.*, 2010). Changes in axonal growth behavior were shown to depend on cis-interactions between IgSF-CAMs as well. Sensory neurons changed their growth cone morphology and growth behavior when axonin1/contactin2 was interacting with NgCAM/L1 (Stoeckli *et al.*, 1996; Kunz *et al.*, 1998). Thus, it is very likely that cis-interactions modulate the signaling downstream of SynCAMs as well (Fig. 2), in line with the observed phenotypes in commissural axon guidance after perturbation of SynCAM expression (Niederkofler *et al.*, 2010).

Concluding remarks

Synaptic cell adhesion molecules do not just glue pre- and postsynaptic elements together; they induce, organize, and modulate synapses. However, a variety of studies have implicated SynCAMs in different functions beyond the synapse. Not only are SynCAM interactions crucial for the myelination of axons in the peripheral and the central nervous system, they also affect early aspects of neural circuit formation by impacting axon guidance. Thus, the link between SynCAM mutations and neurodevelopmental diseases could be more than just aberrant synaptic plasticity. In fact, it is tempting to speculate that the difference between neurodevelopmental diseases could be due to a combination of aberrant synaptic plasticity and additional effects on axonal connectivity in specific circuits. The analysis of brains from patients diagnosed with ASD suggests that there are morphological changes in axonal connectivity in addition to the changes in synaptic function (Geschwind & Levitt, 2007). It remains to be shown whether other families of synaptic cell adhesion molecules have a function beyond the synapse as well.

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Table 1. Different names and functions of the SynCAM family members depending on the context of identification

Names	Function
SynCAM1	Synaptogenesis [1), 2)]
Necl2	Cell adhesion at basolateral membrane of epithelial cells [3), 4)]
TSLC1/ IGSF4	Tumor suppressor [5), 6)]
RA175	Mouse ortholog of TSLC1 [7)]
SgIGSF	Spermatogenesis [8)]
SynCAM2	Synaptogenesis [2)]
Necl3	Cell-cell adhesion [3)]
SynCAM3	Axon terminal-astrocyte contact [9)], myelination in PNS [10), 11)] and CNS [12)]
Necl1	Cell-cell adhesion [3)]
TSLL1	Tumor suppressor [13)]
SynCAM4	Schwann cell-axon contact for PNS myelination [10), 11)]
Necl4	Cell-cell adhesion [3)]
TSLL2	Tumor suppressor [13)]

1) Biederer *et al.*, 2002; 2) Fogel *et al.*, 2007; 3) Takai *et al.*, 2008; 4) Shingai *et al.*, 2003; 5) Kuramochi *et al.*, 2001; 6) Gomyo *et al.*, 1999; 7) Urase *et al.*, 2001; 8) Wakayama *et al.*, 2001; 9) Kakunaga *et al.*, 2005; 10) Maurel *et al.*, 2007; 11) Spiegel *et al.*, 2007 ; 12) Park *et al.*, 2008 ; 13) Fukuhara *et al.*, 2001

Figure 1

Synaptic cell adhesion molecules organize pre- and postsynaptic compartments by specific trans-synaptic interactions.

Synaptic cell adhesion molecules of the SynCAM, the neuroligin/neurexin, and the LRRTM families are sufficient to induce synapses by interaction with specific binding partners in a trans-synaptic manner (Missler *et al.*, 2012). Presynaptic neurexins interact with postsynaptic neuroligins (Südhof, 2008) and LRRTMs (Linhoff *et al.*, 2009). SynCAMs form clusters in cis, before interacting with SynCAM clusters in trans (Fogel *et al.*, 2011). Intracellular, synaptic cell adhesion molecules interact with a variety of scaffold molecules to recruit either the vesicle release machinery presynaptically, or neurotransmitter receptors postsynaptically (Krueger *et al.*, 2012; de Wit *et al.*, 2011; Missler *et al.*, 2012). In the postsynapse, SynCAMs bind to the scaffold molecule CASK, to actin-binding proteins of the 4.1 family and to the guanine nucleotide exchange factor Farp1. Neuroligins and LRRTMs both recruit PSD95 in excitatory synapses. In inhibitory synapses, neuroligins are connected to gephyrin scaffold molecules. N-Cadherin is not sufficient to induce synapses directly but it can induce clustering of neuroligin via the scaffold molecule S-SCAM and stabilize synapses by affecting actin dynamics via β-catenin. In the presynapse both, SynCAMs and neurexins, bind to CASK. CASK in turn forms a complex with Mint1 and Veli.

Figure 2

A complex pattern of SynCAM cis- and trans-interactions affects pathway choices of axons during neural circuit formation.

(A) Axon guidance depends on adhesion molecules. IgSF-CAMs have been implicated in axon guidance at multiple levels. They can keep axons bundled along their trajectory towards an intermediate or the final target (Box 1). At choice points, also called intermediate targets, axon-axon adhesion needs to be downregulated (Box 2) to allow axon-target interaction (Box 3). The interaction with the choice point guides and prepares the growth cone for the next stage of its journey. (B) These different behaviors of axons along their pathway (shown in A) are controlled by a complex interaction pattern between SynCAMs (blue and green represent different SynCAM family members). SynCAMs assemble laterally in cis to form homo- and heterophilic dimers or oligomers. The composition of the clusters in turn may modulate the interaction of SynCAMs in trans (grey arrows). In addition, depending on the composition of the clusters different intracellular signaling cascades are activated.

Figure 1. Synaptic cell adhesion molecules organize pre- and postsynaptic compartments by specific trans-synaptic interactions

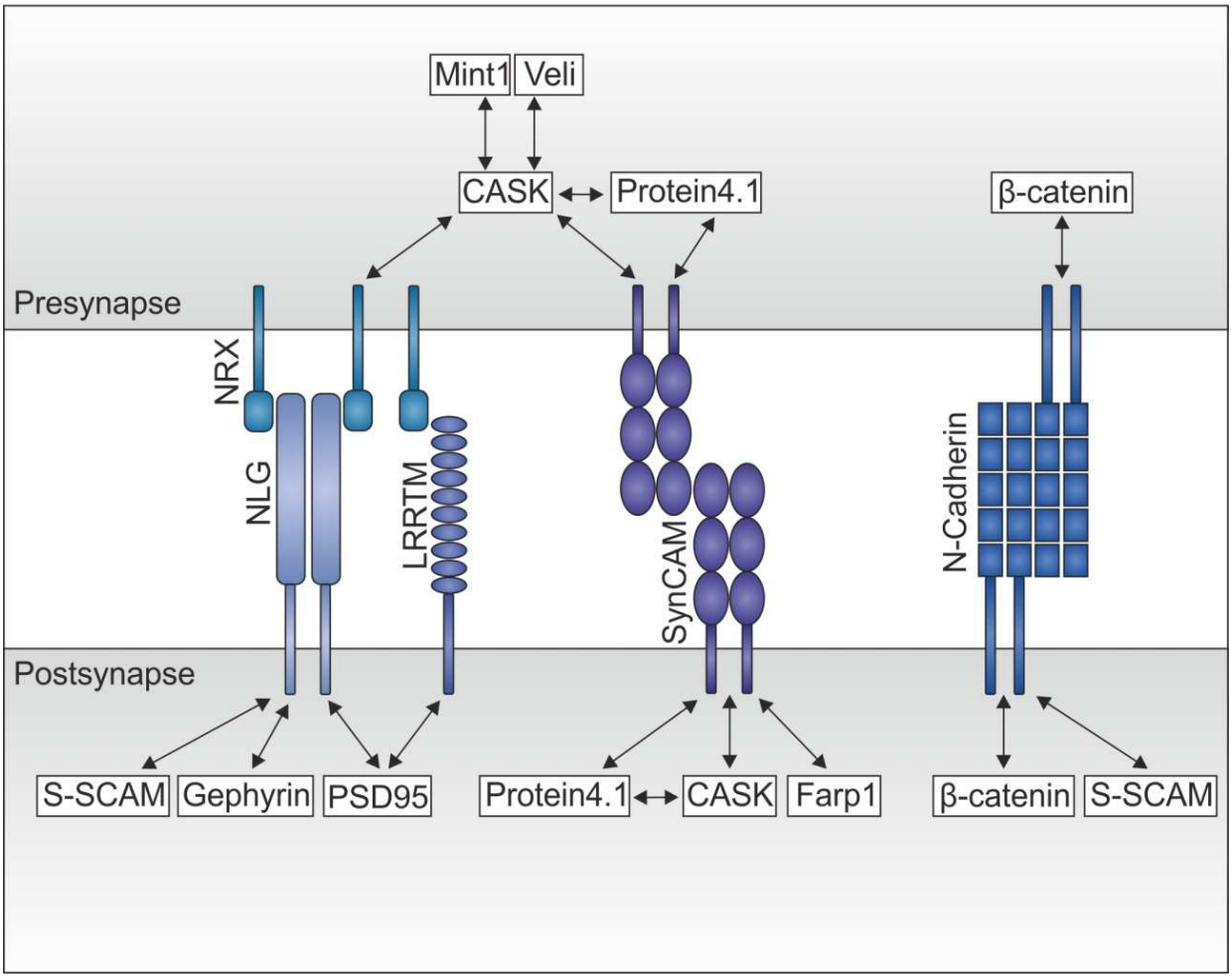
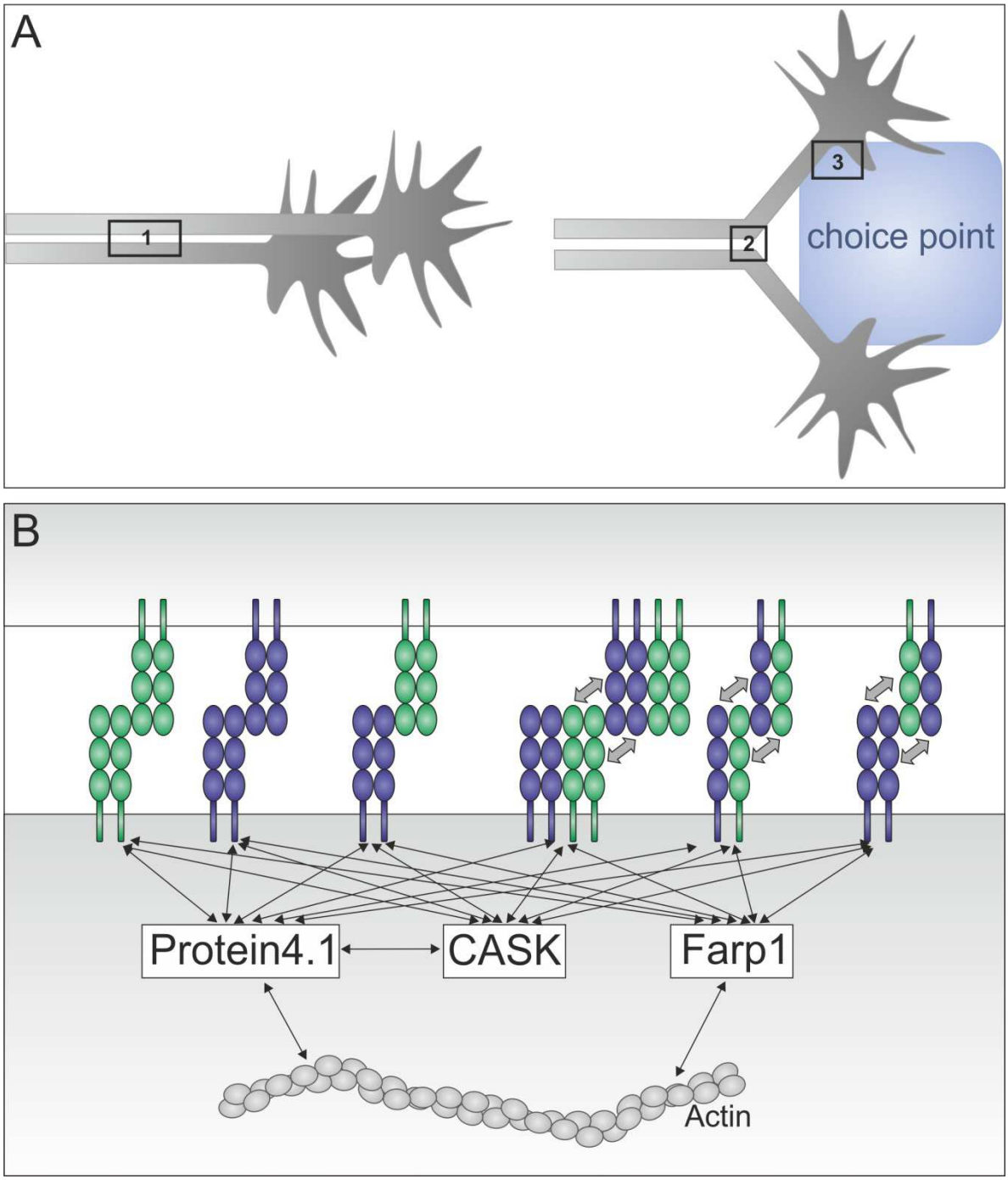


Figure 2. A complex pattern of SynCAM cis- and trans-interactions affects pathway choices of axons during neural circuit formation



Aims of the thesis

SynCAMs, a subgroup of the Ig-superfamily of cell adhesion molecules, have been extensively studied in late steps of nervous system development such as synaptogenesis and myelination. However, their expression during early stages of neural development prompted studies testing a role of SynCAMs in axon guidance. Indeed, previous in vivo studies confirmed a requirement for SynCAMs in commissural axon guidance (Niederkofler *et al.,* 2010). The goal of my PhD thesis was the characterization of the mechanism by which SynCAMs affect guidance. Specifically, the following questions were addressed:

- What is the role of SynCAMs in early sensory neural circuit formation?
- What are the mechanisms underlying the function of SynCAMs in sensory axon pathfinding?
- How do SynCAMs interact with each other? Do they interact via homo- and heterophilic cis- and trans-complexes?

4. Manuscript: The synaptic cell adhesion molecules SynCAMs are involved in early sensory axon pathfinding

The Synaptic Cell Adhesion Molecules SynCAMs Are Involved in Early Sensory Axon Pathfinding

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Abstract

Axonal pathfinding and proper formation of synapses are crucial steps for the development of a functional nervous system**.** SynCAMs, also known as Nectin-like molecules or CADMs, were identified due to their instructive role in synaptogenesis. A detailed analysis of their function implicated SynCAMs in synapse organization and synaptic plasticity. SynCAMs contribute to myelination both in the CNS and in the PNS. In addition to these roles during late stages of neural development, we have demonstrated a requirement for SynCAMs during earlier stages of neural circuit formation. In vivo analysis revealed a role of SynCAM1 and SynCAM2 in post-crossing commissural axon guidance. In addition to the homophilic cis-interaction reported by previous studies, our in vivo results suggested the existence of heterophilic cis-interactions between SynCAM1 and SynCAM2. Indeed, as we show here, the presence of cis-interactions modulated the interaction of SynCAMs with binding partners in trans as observed previously for other Ig-superfamily cell adhesion molecules. These in vitro findings are in agreement with results from in vivo studies, which demonstrated a role for SynCAMs in the formation of sensory neural circuits in the chicken embryo. In the absence of SynCAMs selective axon-axon interactions are perturbed resulting in aberrant pathfinding of sensory afferents.

Introduction

The development of a functional nervous system critically depends on complex processes such as axonal pathfinding, target recognition, and synaptogenesis. The analysis of the molecular mechanisms underlying these different steps suggested common contributors. The synaptic cell adhesion molecules SynCAMs, also known as Nectin-like molecules (Necls) or cell adhesion molecules (CADMs), are a subgroup of the immunoglobulin (Ig)-superfamily of cell adhesion molecules (IgSF-CAMs). SynCAMs were identified based on their role in synapse formation (Biederer *et al.*, 2002). More recently, a role of SynCAM1 was found in synaptic plasticity and spatial learning (Robbins *et al.*, 2010). In agreement with these findings in mice, mutations in SynCAM1 were linked to autism in humans (Fujita *et al.*, 2010; Zhiling *et al.*, 2008) and lack of SynCAM1 impaired social behavior in mice (Takayanagi *et al.*, 2010). Although such deficits are largely associated with synaptic plasticity there is evidence that earlier steps in neural circuit formation may be compromised as well in patients diagnosed with autism or intellectual disability (Stoeckli, 2012). In agreement with the idea that molecules involved in synaptogenesis and synaptic plasticity may also have functions in earlier steps of neural circuit formation we analyzed the role of SynCAMs in axon guidance (Niederkofler *et al.*, 2010). SynCAMs are expressed by dI1 commissural neurons and floor-plate cells during axonal pathfinding. In vivo studies indicated that axonal SynCAMs (SynCAM1 and SynCAM2) and floor-plate SynCAM2 were required for midline crossing and the subsequent rostral turn of commissural axons in the developing chicken spinal cord. Silencing SynCAM2 in floor-plate cells and silencing SynCAM1 or SynCAM2 in commissural dI1 neurons interfered with the correct navigation of their axons along the rostro-caudal axis. Our finding that downregulation of SynCAM2 in commissural neurons also induced pathfinding errors of commissural axons at the floor plate was surprising based on the absence of a significant homophilic trans-interaction of SynCAM2 (Fogel *et al.*, 2007; Niederkofler *et al.*, 2010). Therefore, we postulated that SynCAMs can also form cis-heterodimers in addition to the published cis-homodimers.

In the current study, we tested this hypothesis using SynCAM fusion proteins for in vitro interaction assays. Our results confirm the existence of cis-heterooligomers and suggest a modulatory role for SynCAM cis-interactions on trans-interactions. This in turn affects SynCAM localization on growth cones and axonal interaction patterns, which are the key to the formation of neural circuits. Our in vivo studies demonstrate that SynCAM interactions

are required for axonal fasciculation in the dorsal root entry zone and the extension of sensory afferents along the longitudinal axis of the spinal cord.

Material and Methods

Generation of SynCAM ectodomains and antibodies

Plasmids encoding the ectodomains of chicken SynCAM1 and SynCAM2 fused to AP-myc-6xHis tag of the pAPtag5 vector (Niederkofler *et al.*, 2010) were transfected into HEK293T cells for the generation of SynCAM ectodomains that were used for the binding studies. To purify SynCAMs used as substrate, plasmids encoding the ectodomains of chicken SynCAM1, chicken SynCAM2 and human SynCAM3 were fused to a 6xHis-STOP and cloned into the pAPtag5 vector. The latter ones were also used as antigens to generate polyclonal antibodies against the different SynCAMs. Transfection into HEK293T cells was done using the calcium-phosphate precipitation method. After 24 hours, the medium was changed to serum-free medium (OptiMEM, Gibco). The supernatant containing secreted ectodomains was collected 48 hours later and the SynCAM fusion proteins were purified by affinity chromatography (FPLC) using Ni-NTA agarose beads (Macherey-Nagel). The purity of the ectodomains was confirmed on a silver-stained gel and by Western blotting after SDS-PAGE using mouse anti-myc (supernatant diluted 1:1000; 9E10, Developmental Studies Hybridoma Bank) or rabbit anti-His antibodies (1:10’000, Rockland) and sheep anti-mouse-HRP (1:10’000; Sigma) or goat anti-rabbit-HRP antibodies (1:10’000; Jackson ImmunoResearch), respectively (Fig. 1). Protein concentration was determined using the Bradford method (BioRad Protein Assay, BioRad). Antibodies against the ectodomains of the different SynCAMs were produced by injecting rabbits with 50 µg (SynCAM1 and SynCAM3) and 30 µg (SynCAM2) of the purified antigens. At least three booster injections were given at 6 weeks intervals. Specificity of the antibodies was assessed by Western blot analysis (Fig. 2).

Binding assays

HeLa cells were plated at a density of 20'000 cells per well (1 cm^2) in LabTeks (Nunc). HeLa cells were either single- or co-transfected with full-length pcDNA3.1-SynCAM1-HA or -Flag and pCAGGs-SynCAM2-HA or -Flag constructs (Niederkofler *et al.*, 2010) or empty vectors (pcDNA.3.1-myc/his or pCAGGs) using Lipofectamine 2000 (Invitrogen). The total amount of DNA was the same for single and co-transfection (400 ng/well). Thus, for single transfection, 200 ng of empty vector were co-transfected to get 400 ng total DNA amount. For the trans-binding assay, 60'000 HeLa cells per well (2 cm^2) were plated in a 24-well plate and separately transfected with SynCAM1-HA or -Flag and SynCAM2-HA or -Flag. After 24 hours the different cell populations were mixed 1:1 and plated in LabTeks. SynCAM ectodomains were added 24 hours post-transfection or 24 hours after mixing the differently transfected cell populations at a final concentration of 10 µg/ml in OptiMEM for 90 minutes at 4°C. Cells were gently washed in PBS and fixed in 4% formaldehyde. Cells were permeabilized with 0.1% Triton-X100 for 15 minutes. Antibodies were diluted in 10% fetal calf serum in PBS. Antibodies used for the binding studies were: mouse anti-myc (supernatant diluted 1:10; 9E10, Developmental Studies Hybridoma Bank), rabbit anti-HA (1:2000; Rockland) and goat anti-Flag (1:1000; DDDDK, abcam). For double staining the following secondary antibodies were used: goat anti-mouse-Cy3 (1:250; Jackson ImmunoResearch) and goat anti-rabbit-Alexa488 (1:250; Invitrogen). Secondary antibodies for triple staining were: donkey anti-mouse-Cy3 (1:250; Jackson ImmunoResearch), donkey anti-rabbit-AMCA (1:250; Jackson ImmunoResearch) and donkey anti-goat-Alexa488 (1:250; Invitrogen). For the quantification of the binding strength, random images were taken with a microscope equipped with fluorescence optics (Olympus BX61) and a Hamamatsu ORCA-R2 camera, using the same settings (exposure time, upper/lower limit). The fluorescent intensities of the bound ectodomains (myc-signal) and the intensities of the SynCAM-transfected cells (Flag- and HA-signal) were measured by ImageJ software. To determine the binding strength of ectodomains to single- and co-transfected cells the ratio between the intensity of the ectodomains and the intensity of the transfection was calculated.

Co-immunoprecipitation analysis

HEK293T cells plated in 60 cm^2 dishes were co-transfected with full-length pcDNA3.1-SynCAM1-myc/his or pcDNA3.1-SynCAM1-HA and pCAGGs-SynCAM2-HA or -Flag and empty vectors (pcDNA.3.1-myc/his or pCAGGs) using the calcium-phosphate precipitation method. Twenty-four hours post-transfection, cells were lysed in 150 mM NaCl and 1% Triton-X100 in 20 mM Tris, pH 8.0, and supplemented with protease inhibitor cocktail (Roche). Lysates were incubated with agarose beads coupled to anti-myc (Thermo Scientific), anti-HA (Sigma) and anti-Flag antibodies (Sigma) for 2 hours at 4°C on an orbital shaker. Proteins bound to the anti-myc-matrix were eluted at low pH (pH 2.8) (ProFound c-Myc Tag IP/Co-IP Kit #23620, Thermo Scientific) followed by immediate neutralization with 1 M Tris, pH 9.5, added to a final concentration of 150 mM. Proteins bound to anti-HA- and anti-Flag-matrix were eluted with 100 µg/ml HA- (Sigma) and Flag-peptides (Sigma), respectively. For analysis of co-immunoprecipitated proteins, sample buffer supplemented with 100 mM DTT was added to the samples for SDS-PAGE and Western blotting. Here, the following antibodies were used: mouse anti-myc (supernatant diluted 1:1000; 9E10, Developmental Studies Hybridoma Bank), rabbit anti-HA (1:20'000; Rockland), goat anti-Flag (1:10'000; DDDDK, abcam), sheep anti-mouse-HRP (1:10'000; Sigma), goat anti-rabbit-HRP (1:10'000; Jackson ImmunoResearch), rabbit anti-goat-HRP (1:10'000; ICN/Cappel). For transfection control, cells were lysed in total lysis buffer containing 2% SDS, 10% glycerol, 6 M urea and 5% mercaptoethanol in 62.5 mM Tris, pH 6.8.

Chemical cross-linking assay

HEK293T cells cultured in 60 cm^2 dishes were co-transfected with pcDNA3.1-SynCAM1-HA and pcDNA3.1-SynCAM1-Flag or pcDNA3.1-SynCAM1-HA and pCAGGs-SynCAM2-Flag using the calcium-phosphate precipitation method. After 24 hours, cells were washed in PBS and detached with 5 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS, pH 8.0. Cells were dissociated by trituration to obtain single cells and plated at a density of 300'000 cells per 10 cm^2 dish. Six hours later cells were carefully washed in PBS, pH 8.0, on ice. For cross-linking, 1 mM bis-sulfosuccinimidyl suberate (BS^3; Thermo Scientific) in H_2O was added to the cells and incubated for 1 hour at 4°C while gently shaking. The reaction was quenched by adding 1 M Tris, pH 7.5, to a final concentration of 20 mM for 15 minutes at RT. Cells were lysed in 150

mM NaCl and 1% Triton-X100 in 20 mM Tris, pH 8.0, and supplemented with protease inhibitor cocktail (Roche). To co-immunoprecipitate cross-linked SynCAMs the lysates were incubated with anti-HA antibody coupled agarose beads (Sigma) for 2 hours at 4°C on an orbital shaker. Proteins bound to the beads were eluted in non-reducing sample buffer by heating samples to 95°C for 5 minutes. For analysis of cross-linked proteins, 100 mM DTT was added to samples followed by SDS-PAGE and Western blotting using rabbit anti-HA (1:20'000; Rockland), goat anti-Flag (1:10'000; DDDDK, abcam), goat anti-rabbit-HRP (1:10'000; Jackson ImmunoResearch) and rabbit anti-goat-HRP (1:10'000; ICN/Cappel).

Preparation of RNA probes for in situ hybridization

In situ hybridization probes for the detection of chicken SynCAM mRNA were produced from ChEST583g11 (SynCAM1), ChEST114o11 (SynCAM2) and CHEST478g10 (SynCAM3) obtained from Source Bioscience (Cambridge, UK). DIG-labeled probes were generated by in vitro transcription with the DIG-RNA labeling mix (Roche) as described previously (Mauti *et al.*, 2006). Embryos were sacrificed at the desired Hamburger and Hamilton stage (Hamburger & Hamilton, 1951), subjected to cryoprotection and cut into 25-µm-thick sections. Probes (0.75 ng/µl) were hybridized on chicken spinal cord cross sections as described previously (Mauti *et al.*, 2006).

In vitro assays with dissociated sensory neurons

Chicken embryos were sacrificed at E5 (HH25/26) and E8 (HH34). DRGs were dissected and collected in ice-cold PBS. To dissociate DRGs 0.25% trypsin was added and DRGs were incubated for 10 to 15 minutes at 37°C. Cells were resuspended in defined medium (see below) and gently triturated to get single neurons. Dissociated sensory neurons were cultured in MEM with GlutaMAX (Gibco), supplemented with N3 (100 µg/ml transferrin, 10 µg/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 200 ng/ml corticosterone, 200 µM putrescine, 60 nM sodium selenite), 20 ng/ml NGF (Invitrogen) and 4 mg/ml Albumax (Invitrogen).

LabTeks were precoated with 10 µg/ml poly-L-lysine (Sigma) followed by coating with either 10 µg/ml laminin (Invitrogen), 10 µg/ml SynCAM1^{ecto} or 10 µg/ml SynCAM2^{ecto} for two hours at 37°C. Dissociated sensory neurons were cultured at a density of 10'000 to 20'000 cells per well for 48 hours. For surface staining, antibodies were directly added to the medium for 45 minutes at 4°C. Cells were gently washed with PBS and fixed in 4% formaldehyde. Antibodies used for immunofluorescence staining were: rabbit anti-SynCAM1 (1:300), rabbit anti-SynCAM2 (1:100-1:300), mouse anti-axonin1 (1:150; see PhD thesis Kunz B., 1996: Function blocking monoclonal antibodies against the neural cell adhesion molecule axonin1), donkey anti-rabbit-Cy3 (1:250; Jackson ImmunoResearch) and goat anti-mouse-Alexa488 (1:250; Invitrogen). Pictures were taken with a fluorescence microscope (Olympus BX61) and a Hamamatsu ORCA-R2 camera.

For the choice assay, COS7 cells were plated on LabTeks at a density of 15'000 cells per well and transfected with full-length pcDNA3.1-SynCAM1-HA, pCAGGs-SynCAM2-HA, pcDNA3.1-humanSynCAM3-HA and pcDNA3-MARCKS-GFP (kind gift of Silvia Arber) using Lipofectamine 2000 (Invitrogen). After 24 hours, 2'000 dissociated sensory neurons were added to the COS7 cell layer and cultured for 24 hours. After fixation in 4% formaldehyde, cells were stained with rabbit anti-HA (1:2000; Rockland), mouse anti-neurofilament (1:1500; RMO270, Invitrogen), goat anti-GFP-FITC (1:500; Rockland), goat anti-rabbit-Alexa488 (1:250; Invitrogen) and goat anti-mouse-Cy3 (1:250; Jackson ImmunoResearch). Images were taken randomly with a fluorescence microscope (Olympus BX51) and an Olympus XC30 camera. For quantification, the number of sensory axon tips ending on a transfected cell was counted and divided by the total number of axon tips. This ratio was normalized to the transfected area (measured with ImageJ software).

For the outgrowth assay poly-L-lysine-precoated LabTeks (10 µg/ml) were coated with 0.4 µg/ml, 10 µg/ml and 50 µg/ml SynCAM1^{ecto}, SynCAM2^{ecto}, SynCAM3^{ecto} or Albumax (Gibco) as described above. To obtain low density cultures 4'000 dissociated sensory neurons per well were cultured. After 28 or 48 hours neurons were fixed in 4% formaldehyde and visualized by neurofilament staining using mouse anti-RMO270 (1:1500; Invitrogen) and goat anti-mouse-Cy3 (1:250; Jackson ImmunoResearch). Pictures were taken randomly with a fluorescence microscope (Olympus BX51) and an Olympus XC30 camera. For quantification of neurite lengths the longest branch from the cell body to the axon tip and the sum of all

neurites of one neuron (total axon length per neuron) were measured using CellM software (Olympus). Only isolated neurites with no contact to other neurons were considered for analysis.

In ovo RNA interference

In ovo RNA interference (RNAi) was used to silence genes of interest as described previously (Pekarik *et al.*, 2003). In brief, a solution containing 300 ng/µl of long dsRNA together with a reporter plasmid containing GFP under the control of the β-actin promotor (20 or 50 ng/µl) was injected into the central canal of E2 chicken embryos (HH12-HH15) to efficiently target the dorsal root ganglia (DRGs). Embryos were electroporated with 5 pulses of 18 Volts and 50 ms length with 1-second interpuls intervals. For the generation of long dsRNA the following ChESTs obtained from Source Bioscience (Cambridge, UK) were used: ChEST583g11 (SynCAM1), ChEST96i3 (SynCAM2) and CHEST478g10 (SynCAM3). DsRNA was prepared by in vitro transcription with unlabeled rNTPs followed by hybridization of sense and antisense RNA strands as previously described (Pekarik *et al.*, 2003). All dsRNAs were recognizing a target sequence of 600-800 bps in the 3'UTR of the corresponding SynCAM mRNA. Embryos were sacrificed at HH24.5/HH25 for whole-mount preparations and at HH25/26 for vibratome sectioning and cultures of DRG explants. The efficiency and specificity of the dsRNA was demonstrated previously (Niederkofler *et al.*, 2010). To reconfirm this for our approach, the efficiency and specificity of SynCAM knockdown by RNAi was tested in vitro (Fig. 3A-M). For this purpose long dsRNA against the different SynCAMs was digested into siRNA by ShortCut RNase III (New Enlgand BioLabs) for 20 minutes at 37°C. Successful digestion was confirmed by gel electrophoresis. HEK293T cells were plated at a density of 40'000 cells per LabTek-well and triple-transfected using Lipofectamine 2000 (Invitrogen) with either 50 ng/well pCAGGs-destabilized-GFP fused to SynCAM1-3'UTR, SynCAM2-3'UTR or SynCAM3-3'UTR (Niederkofler *et al.*, 2010), 50 ng/well of the different siRNAs and 50 ng/well of a construct encoding tomato-fluorescent protein as transfection control. After 24 hours, cells were fixed in 4% formaldehyde. Random pictures were taken with constant settings (exposure time, upper limit) using a fluorescent microscope (Olympus BX51) and an Olympus XC30 camera. For quantification the intensity of the GFP signal was normalized to the intensity of the Tomato signal. Intensities were measured with the ImageJ software.

To test the efficiency of dsRNA in vivo, 1 µg/µl dsSynCAM1 together with 20 ng/µl GFP was injected and electroporated in three-day-old embryos (HH17-HH19). Embryos were sacrificed at E5 (HH25/HH26) and commissural neurons and motoneurons were separately isolated. The cells were lysed in buffer containing 180 mM NaCl, 5 mM EDTA and 1% Triton-X100 in 50 mM Tris, pH 8.0, supplemented with protease inhibitor cocktail (Roche). Downregulation was analyzed by SDS-PAGE followed by Western blotting using rabbit anti-SynCAM1 (1:10'000), rabbit anti GAPDH (1:2500; abcam) and goat anti-rabbit-HRP (1:10'000; Jackson ImmunoResearch) (Fig. 3N). For quantification of the knockdown, the intensities multiplied by the areas of the bands were measured using ImageJ software (Fig. 3O). The signal of SynCAM1 was normalized to the signal of GAPDH.

Preparation of intact DRG explants for scanning EM analysis

For the analysis of neuronal networks, the number of filopodia and the morphology of growth cones, intact chick DRGs (E8) were cultured on 12 mm diameter round poly-L-lysine-precoated (10 µg/ml) coverslips, coated with 10 µg/ml SynCAM1^{ecto}, SynCAM2^{ecto}, SynCAM3^{ecto}, Albumax or laminin. For the analysis of DRGs lacking SynCAMs, in ovo RNAi in E2 chicken embryos was performed as described above. DRGs (E5) transfected with 500 ng/µl of either dsSynCAM1, dsSynCAM2 or dsSynCAM3 together with 50 ng/µl β-actin-GFP and DRGs transfected with 50 ng/µl β-actin-GFP only were cultured on 12 mm coverslips coated with collagen (66.7 µg/ml; Millipore). After 48 hours, DRGs were fixed in 2.5% glutaraldehyde and 0.8% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 minutes to one hour at RT or overnight at 4°C. Samples were incubated with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 30 minutes on ice followed by dehydration in a graded series of ethanol from 70% to 100%. For scanning EM analysis samples were prepared by critical point drying followed by Platinum/Carbon coating. Pictures were taken with a scanning electron microscope (Zeiss Supra 50 VP). Images were taken from the periphery of the axonal network. For calculation of the number of filopodia per neurite, filopodia along single neurites were counted and normalized to the length of the neurite using the CellM software (Olympus). To calculate the percentage of branched filopodia, all filopodia having at least a branch of second order were counted. The area of growth cones was measured with the CellM software (Olympus). The number of growth cone filopodia was counted and

normalized to the growth cone area. Growth cones were classified into four different shapes: round, finger-like, long and thin, and long and flat.

Immunohistochemistry on cryostat and vibratome sections

For immunohistochemistry analysis, either 25-µm-thick cryostat sections of HH25/26 chicken and E12.5 mouse spinal cords or 250-µm-thick vibratome sections of HH25/26 chicken spinal cords were prepared. Cryo- and vibratome sections were permeabilized with 0.1% and 0.3% Triton-X100, respectively. Antibodies were diluted in 10% fetal calf serum in PBS and used 1:10'000-1:20'000 for staining with rabbit anti-SynCAM1, 1:1000 for staining with rabbit anti-SynCAM2 and 1:250 for staining with rabbit anti-SynCAM3. Vibratome slices were stained with rabbit anti-axonin1 (1:10'000; Ruegg *et al*., 1989). For all stainings donkey anti-rabbit-Cy3 (1:250; Jackson ImmunoResearch) was used as secondary antibody.

Neurofilament staining of whole-mount embryos

Whole-mount embryos were prepared as described previously (Mauti *et al.*, 2007). In brief, experimental embryos injected with dsRNA against SynCAM1, SynCAM2 and SynCAM3 and control embryos injected with β-actin-GFP or untreated control embryos were sacrificed at HH24.5/HH25, permeabilized with 1% Triton-X100 for 1 hour, incubated with 20 mM lysine in 0.1 M sodium phosphate, pH 7.4, and incubated in 10% fetal calf serum in PBS. Primary anti-neurofilament antibody (1:1500; RMO270, Invitrogen) and secondary goat anti-mouse-Cy3 antibody (1:250; Jackson ImmunoResearch) were used to visualize peripheral nerves after dehydration in a graded series of methanol from 25% to 100% and storage in benzyl benzoate/benzyl alcohol (2:1).

Quantification of the sensory axon bundle in whole-mount preparations and vibratome sections

The longitudinal axon bundle formed by sensory afferents along the neural tube and the DRGs were analyzed in whole-mount embryos using a binocular equipped with fluorescence (Olympus SZX12) and a KAPPA CF8/4 camera. Embryos were classified as having no (homogenous dorsal sensory axon bundle), a weakly (tendency to variable thickness) or a strongly aberrant phenotype (wavy structure of dorsal sensory axon bundle with inhomogeneous thickness) and the respective percentages were calculated. Segmentation of the sensory axon bundle in vibratome sections was analyzed using a microscope equipped with fluorescence optics (Olympus BX51) and an Olympus XC30 camera. The percentage of sections showing segmentation of the axon bundles per experimental and per control group was calculated. The analysis was done by an observer who was blind to the treatment group. For statistical analysis two-tailed Fisher exact probability test was used.

For the quantification of the thickness of the sensory axon bundle we used pictures of the dorsal spinal cord and DRGs of whole-mount embryos that were taken with a binocular equipped with fluorescence optics (Olympus SZX12) and a KAPPA CF8/4 camera. The thickest region of the sensory axon bundle at the level of the roots and the thinnest region of the sensory axon bundle in the region between two DRGs was measured using ImageJ software. The thickness at the thinnest region was divided by the thickness at the thickest region. The average of the ratios per embryo and per group was calculated and compared to the ratio of GFP-injected control embryos.

Statistical analysis

For statistical analysis the two-tailed Student's t-test or one-way ANOVA followed by the Tukey HSD post-hoc test were used to calculate p-values using Microsoft Excel 2007 or vassarstats.net, respectively. P-values lower than 0.05 were considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Values represent the mean; error bars represent the standard error of the mean (s.e.m.).

Results

SynCAMs interact in a complex pattern of homophilic and heterophilic cis- and trans-interactions

Results from our recent in vivo study suggested the existence of SynCAM1/SynCAM2 hetero-cis-dimers on commissural dI1 axons (Niederkofler *et al.*, 2010). We found that silencing SynCAM2 expressed by floor-plate cells resulted in pathfinding errors of axons at the floor-plate exit site. Similarly, silencing SynCAM1 or SynCAM2 in dI1 commissural neurons resulted in the same axonal navigation problems at the floor-plate exit site. While the results obtained after blocking SynCAM1 were explained by its strong heterophilic trans-interaction to floor-plate SynCAM2, the finding that silencing SynCAM2 on dI1 axons also interfered with pathfinding was surprising, as homophilic SynCAM2 interactions were found to be very weak (Niederkofler *et al.*, 2010). Thus, we concluded that the most parsimonious explanation for the observed phenotype was the existence of SynCAM1/SynCAM2 cis-dimers or cis-oligomers on dI1 axons.

To find evidence for heterophilic cis-interactions, we carried out binding assays where purified tagged ectodomains of SynCAM1 and SynCAM2 were added to HeLa cells expressing full-length SynCAMs. First, we verified the previously reported difference between homo- and heterophilic interaction strengths between SynCAM1 and SynCAM2 (Fogel *et al.*, 2007; Niederkofler *et al.*, 2010). In line with those studies, homophilic interactions were very weak in comparison to heterophilic interactions between SynCAM1 and SynCAM2 (Fig. 4). We observed strong binding of SynCAM1^{ecto} to SynCAM2-transfected cells (Fig. 4C) and of SynCAM2^{ecto} to SynCAM1-transfected cells (Fig. 4B). In contrast, homophilic binding between soluble SynCAM1^{ecto} and SynCAM1-expressing cells (Fig. 4A) and SynCAM2^{ecto} and SynCAM2-expressing cells (Fig. 4D) was weak. These results were partly confirmed by co-immunoprecipitation studies (Fig. 4G-I). Lysates from HEK293T cells co-transfected with full-length SynCAM1 tagged with HA or myc and full-length SynCAM2 tagged with HA or Flag were subjected to co-immunoprecipitation using either anti-HA-, anti-myc- or anti-Flag-antibody-coupled agarose beads. We were able to demonstrate strong heterophilic SynCAM1-SynCAM2-binding (Fig. 4I) and weak homophilic SynCAM2 interactions (Fig. 4H). However, the homophilic SynCAM1 interaction was well detectable (Fig. 4G), which was in contrast to the weak binding of SynCAM1^{ecto} to SynCAM1-transfected cells (Fig. 4A).

In line with observations made for other IgSF-CAMs (Kunz *et al.*, 1998), we speculated that heterophilic cis-interactions might alter the affinity for trans-interactions. To test the possibility of change in trans-binding behavior of SynCAMs depending on the formation of hetero- versus homophilic cis-clusters, we co-transfected HeLa cells with both SynCAMs, HA- or Flag-tagged, and incubated the cells with either SynCAM1 or SynCAM2 ectodomains (Fig. 5A-E). To compare the binding strengths of the ectodomains to co-transfected cells, we included the homophilic and heterophilic combinations in this assay. Again, heterophilic binding of SynCAM1^{ecto} to SynCAM2 (Fig. 5A) and SynCAM2^{ecto} to SynCAM1 (Fig. 5B) was much stronger than the homophilic binding of SynCAM1 and SynCAM2 (Fig. 5E). Binding of SynCAM1^{ecto} to cells co-transfected with SynCAM1 and SynCAM2 was reduced by 52% compared to binding of SynCAM1^{ecto} to SynCAM2-single expressing cells (Fig. 5C, E). An even stronger reduction by 91% was observed for SynCAM2^{ecto} trans-binding to co-transfected cells compared to SynCAM2^{ecto} binding to SynCAM1-single expressing cells (Fig. 5D, E). Taken together, our in vitro binding studies demonstrate that the presence of both SynCAM1 and SynCAM2 in the cell membrane reduces or even inhibits the binding of SynCAM ectodomains, suggesting that the formation of a heterophilic SynCAM1/SynCAM2 cis-dimer weakens the trans-binding of SynCAMs.

To rule out the possibility that the observed reduction of SynCAM trans-binding to co-transfected cells was caused by the unavailability of binding partners on the cell surface due to their recruitment into heterophilic interactions at cell-cell contact sites, we carried out another series of binding studies. For this purpose, HeLa cells were separately transfected with HA- and Flag-tagged SynCAM1 and SynCAM2, respectively. The two cell populations were mixed and incubated with either SynCAM1 or SynCAM2 ectodomains (Fig.5 F-M). Under these conditions, we analyzed the binding of SynCAM1 and SynCAM2 ectodomains to groups of cells expressing both, SynCAM1 and SynCAM2, in comparison to groups of cells only expressing either SynCAM1 or SynCAM2. At the concentrations used in our binding assays, the soluble SynCAM ectodomains added to the cultures were not able to compete with cell surface SynCAMs engaged in heterophilic cell-cell contact sites (Fig. 5F, G, J, K; open arrowheads). However, they still bound to SynCAMs on the cell membrane, which were not recruited to these contact sites (Fig. 5F, G, J, K; white arrowheads). These results indicate that not all of the SynCAM molecules on the cell surface were recruited to engage in

heterophilic cell-cell contacts and, thus, reduced trans-interactions were not due to the absence of SynCAM binding partners on the cell surface at non-contact sites.

More direct evidence for the existence of heterophilic SynCAM1/SynCAM2 cis-interactions was obtained by cross-linking experiments (Fig. 5N, O). We added bis-sulfosuccinimidyl suberate (BS^3), an 11Å-long cross-linker, to cells co-transfected with SynCAM1-HA/SynCAM1-Flag and SynCAM1-HA/SynCAM2-Flag. Co-transfection of SynCAM1-HA and SynCAM1-Flag served as positive control as it is known that SynCAM1 forms homophilic cis-complexes (Fogel *et al.*, 2011). To make sure that trans-interactions did not confound our results, we replated transfected cells at low density to obtain single cell cultures. Under these conditions, immunoprecipitation of SynCAM1-HA with anti-HA-matrix would only pull-down SynCAM1-Flag and SynCAM2-Flag, respectively, if they were covalently bound to each other by cross-linking. Indeed, SynCAM1-HA and SynCAM1-Flag as well as SynCAM1-HA and SynCAM2-Flag were successfully cross-linked into higher molecular weight oligomers (Fig. 5N, O, lanes 5 and 6). Interestingly, no dimers were formed suggesting that SynCAMs assemble into higher multimeric complexes.

Taken together, these findings confirm the presence of SynCAM1-SynCAM2 heterophilic cis-interactions. Furthermore, our results suggest that hetero-cis-clusters modulate the binding preferences in trans, as trans-binding of SynCAM1 and SynCAM2 to heterophilic cis-clusters was strongly reduced or virtually abolished.

SynCAMs are expressed in DRG sensory neurons throughout development

Prior to our findings that SynCAMs are required for commissural axon navigation at the spinal cord midline (Niederkofler *et al.*, 2010), they had only been implicated in later stages of neural circuit formation (Biederer *et al.*, 2002; Maurel *et al.*, 2007; Robbins *et al.*, 2010; Spiegel *et al.*, 2007). However, our expression analysis suggested that SynCAMs could be involved in early aspects of sensory neuron development in the PNS as well. We found SynCAM1 in somites at HH11 (Fig. 6A). At HH18 when dorsal root ganglia (DRGs) start to form, we detected both SynCAM1 and SynCAM2 expression (Fig. 6G, H). At this stage all SynCAMs were expressed in motoneurons (Fig. 6G-I). At HH21, all three SynCAMs were also expressed in DRGs (Fig. 6J-L). Staining at HH23 showed that SynCAMs were present in the commissural neurons in the dorsal spinal cord (Fig. 6M-O). Furthermore, SynCAM2 and SynCAM3 showed strong staining in the floor plate throughout all stages analyzed. SynCAM1 expression in the floor plate was weaker and no longer detectable at HH23. Expression of all SynCAMs in the DRGs persisted until later stages. At HH30 and HH34, SynCAMs were expressed in a subtype-specific manner in DRG neurons (Fig. 6S-T). SynCAM1 was restricted to nociceptive neurons in the dorsomedial region, whereas SynCAM2 was mainly found in ventrolaterally located proprioceptive neurons. Between HH30 and HH34 SynCAM3 was present throughout the DRGs, although at different expression levels (Fig. 6U).

We next tested the expression of SynCAM1 and SynCAM2 protein using polyclonal antibodies (Fig. 6V-X). At HH26, SynCAM1 and SynCAM2 protein were detectable in a pattern that was in agreement with the localization of the mRNA. SynCAM1 was found mainly in the dorsomedial DRG and on sensory axons (Fig. 6V). Strong immunoreactivity was also observed in the roof plate and on commissural axons both in the commissure and on post-crossing axons, in the notochord and in cells of the dermomyotome. SynCAM2 immunoreactivity was observed in the DRG, most strongly in the ventrolateral part (Fig. 6W). Sensory axons and commissural axons, both in the commissure and in the longitudinal axis, were positive for SynCAM2. In contrast to SynCAM1, SynCAM2 was found in the floor plate, in line with the results of the in situ hybridization study. The antibody against human SynCAM3 did not recognize the chicken protein (data not shown). Staining of mouse tissue confirmed expression in DRGs and motoneurons but failed to detect SynCAM3 in the floor plate (Fig. 6X).

To investigate whether SynCAM1 and SynCAM2 were also expressed on sensory growth cones we cultured dissociated sensory neurons of 5-day-old chicken embryos on a laminin coated surface and incubated them for two days. Staining was carried out on unfixed cells to only visualize the protein on the surface of axons and growth cones. Both, SynCAM1 (Fig. 6Y) and SynCAM2 (Fig. 6Z) were present on the surface of sensory axons and growth cones with prominent expression in growth cone filopodia, as indicated by colocalization with axonin (Fig. 6Z, inset).

In summary, SynCAMs are found in sensory neurons of the dorsal root ganglia throughout development, suggesting a function in sensory neural circuit formation. In support of this hypothesis, SynCAM1 and SynCAM2 proteins were found on the surface of sensory axons and growth cones in vitro.

SynCAMs mediate adhesion of sensory axons

We started investigating the function of SynCAMs in sensory neural circuit formation with a series of in vitro experiments. First, we demonstrated an adhesive effect of SynCAMs on DRG sensory neurons in an in vitro choice assay. Dissociated sensory DRG neurons were cultured on a layer of COS7 cells expressing SynCAMs for 24 hours (Fig. 7A-D). We calculated the proportion of growth cones on SynCAM-expressing cells and compared the values to control-transfected, GFP-expressing cells (MARCKS-GFP). For cells dissected from E5 embryos, we found 4.6-fold more growth cones on SynCAM1-, 3.7-fold more growth cones on SynCAM2- and 3.5-fold more growth cones on SynCAM3-expressing cells compared to cells transfected with MARCKS-GFP (Fig. 7E). In case of neurons dissected from E8 embryos, 3.2-fold more growth cones were found on SynCAM1-, 2.7-fold more growth cones on SynCAM2-, and 2.2-fold more growth cones on SynCAM3-transfected cells (Fig. 7F). Thus, neurons dissected from 5-day-old and from 8-day-old embryos showed a strong preference for SynCAM-expressing cells.

SynCAMs induce neurite outgrowth of old but not young sensory neurons

Cell adhesion molecules of the Ig-superfamily have been shown to promote neurite growth of sensory neurons (Buchstaller *et al.*, 1996; Kuhn *et al.*, 1991; Lustig *et al.*, 1999; Morales *et al.*, 1993; Stoeckli *et al.*, 1991; Stoeckli *et al.*, 1996). To test whether this was also true for the SynCAM subgroup, we cultured dissociated sensory neurons dissected from 5-day-old and from 8-day-old embryos at low density on SynCAMs and control substrates (PLL and Albumax). Neurite lengths were measured after 28 hours and 48 hours on three different concentrations of purified His-tagged SynCAM ectodomains (Fig. 8). E5 sensory neurons cultured on 50 µg/ml SynCAM substrate showed a tendency to longer total axon lengths on SynCAM2 and increased values for the longest axon per neuron on both, SynCAM1 and SynCAM2, compared to poly-L-lysine (PLL) substrate (Fig. 8A, B). However, no effect of SynCAM substrate on axon elongation was seen when the concentration of coated proteins was decreased to 10 µg/ml or 0.4 µg/ml (Fig. 8C-F). E5 neurons did not show a dose-dependent response to SynCAM substrates as axons were of similar length on all concentrations (Fig. 8G). This was in contrast to the behavior of E8 neurons. SynCAM substrate significantly promoted elongation of older sensory axons. SynCAM2 and SynCAM3 exerted the biggest effect on neurite outgrowth as the total axon length as well as the length of the longest axon were significantly increased compared to PLL substrate at all SynCAM concentrations (Fig. 8H-M). The promotion of axon growth was concentration-dependent. The increase in concentration of SynCAM2 and SynCAM3 correlated with the increase in axon length (Fig. 8N). SynCAM1 substrate only had a minor effect on axon outgrowth although neurites showed a tendency to get longer when compared to control substrates (Fig. 8H-M). In contrast to SynCAM2 and SynCAM3, this effect was concentration-independent as axons grown on SynCAM1 substrate were of similar length (Fig. 8N). When axon outgrowth of E8 sensory neurons was stopped after 28 hours the values for the total axon length as well as the length of the longest axon per neuron were significantly increased on all SynCAMs but independent of the coated concentration (Fig. 8O-U). Thus, the concentration-dependent effects of SynCAM2 and SynCAM3 on axon lengths are only detectable after 48 hours but an outgrowth-promoting effect is also seen at 28 hours.

Together, these results show that SynCAMs do not have a strong effect on axon elongation of young sensory neurons but they promote axon outgrowth later in development.

SynCAMs influence axon-axon interactions

The analysis of sensory neurons grown on SynCAM substrates revealed a different morphology compared to neurons grown on laminin or poly-L-lysine. For a more detailed assessment of the behavior of sensory neurites, we cultured intact DRGs as explants on different SynCAM substrates and analyzed these cultures at the electron microscopic level (Fig. 9).

Axons grown on SynCAM1, SynCAM2 and SynCAM3 substrate showed a 1.6-fold, 1.4-fold and 1.5-fold increase in the number of filopodia branching off a neurite compared to axons grown on PLL (Fig. 9A-G). Moreover, filopodia of axons grown on SynCAM substrates showed about 10% more higher order branching compared to filopodia on PLL, Albumax and laminin (Fig. 9H). In line with these measurements, DRG explants formed highly disorganized axonal networks on SynCAM substrates (Fig. 9I-N). Instead of radially arranged, parallel axon bundles as seen on laminin, Albumax and PLL, the network on SynCAM substrates was highly disorganized with axons frequently crossing between bundles.

To test whether not only externally added but also endogenous SynCAMs are able to influence the fasciculation pattern of axons, we cultured DRGs lacking SynCAMs and control DRGs on collagen and compared the morphology of axonal networks. We also found striking differences in axonal fasciculation behavior (Fig. 9O-S). Again, we observed a high degree of neurite crossing between axon bundles in the absence of any one of the SynCAMs resulting in more connections between bundles and a disorganized morphology of the axonal network.

Taken together, our in vitro analysis of DRG explant cultures demonstrated a change in axonal network morphology when DRGs were grown on SynCAM substrates compared to laminin, Albumax or PLL. A similar finding was observed after knockdown of SynCAMs in DRG neurites. Thus, adding SynCAMs externally or decreasing endogenous SynCAM levels results in altered axon-axon contacts resulting in a higher degree of axonal crossing and connections between bundles.

SynCAM substrates affect growth cone morphology and SynCAM distribution on the growth cone surface

The observed changes in axon-axon contact in our DRG explant cultures were reflected by changes in growth cone morphologies. On SynCAM substrates, growth cones were significantly larger compared to laminin, PLL or Albumax (Fig. 10A-G). They reached an average area of 396.6 μm^2 on SynCAM1, 799.7 μm^2 on SynCAM2 and 575.9 μm^2 on SynCAM3. In contrast, the size of growth cones grown on PLL was on average 244.7 μm^2, on Albumax 149.1 μm^2 and on laminin 133.8 μm^2. Furthermore, on SynCAM2 substrate the number of filopodia normalized to the growth cone area was significantly reduced compared to control substrates (Fig. 10H). The reduction in the number of growth cone filopodia on SynCAM1 and SynCAM3 substrate was not significantly different to the control substrates. Not only the area but also the shape of the growth cones differed depending on the substrate (Fig. 10I). On SynCAM2 substrate 77% of the growth cones revealed a round and 23% a long and flat shape, whereas growth cones on SynCAM1 and SynCAM3 were mostly round (54% and 60%, respectively) or of finger-like morphology (26% and 27%, respectively). Growth cones on PLL were also mostly of round (53%) and of finger-like shape (35%), similar to growth cones on SynCAM1 and SynCAM3, but they were much smaller. Most of the growth cones on laminin and Albumax showed a finger-like morphology (51% and 54%, respectively). These results show that growth cones respond differently to SynCAM substrates, reflected in a change in size and shape.

As we observed that growth cone morphology changed upon contact with SynCAM substrates we next investigated whether SynCAMs expressed on the growth cone membrane were actively involved in the contact with the substrate. To this end, we compared surface staining of SynCAM1 and SynCAM2 on sensory growth cones cultured on SynCAM1, SynCAM2 or laminin substrate. Live staining of axons grown on SynCAM1 and SynCAM2 substrate showed that SynCAM1 was cleared from the apical growth cone surface (Fig. 10J, J', K, K'). Similarly, SynCAM2 was depleted from the apical growth cone surface on both SynCAM1 and SynCAM2 substrate (Fig. 10M, M', N, N'). In contrast, both SynCAM1 and SynCAM2 were readily detected on the apical growth cone surface on laminin (Fig. 10L, L', O, O'). The depletion of SynCAMs from the apical surface indicates that they are redistributed to the substrate-facing surface of the growth cone in a substrate-dependent manner. On

laminin substrate, where axons grow in an integrin-dependent manner, SynCAMs are not recruited to the substrate-facing surface of the growth cone.

Taken together, the changes in growth cone morphology and the redistribution of SynCAMs to the growth cone membrane facing the substrate suggests an active contribution of these molecules to the behavior of growth cones and axons.

SynCAMs are required for the proper spinal cord entry of sensory afferents

Taken together, our in vitro analyses suggested a role of SynCAMs in regulating axon-axon contacts during sensory neural circuit formation. A good opportunity to test such a function in vivo is the entry of sensory axons into the dorsal spinal cord. When axons enter the dorsolateral spinal cord at the dorsal root entry zone (DREZ), they need to select between a more ventral and a more dorsal pathway to reach the appropriate position in the dorsal funiculus, from where they extend collaterals (Eide & Glover, 1995). Within this longitudinal bundle axons sort out depending on their sensory modality. Proprioceptive axons bifurcate in a rather Y-shaped angle to reach the dorsomedial funiculus, whereas nociceptive axons extend in a T-shaped manner, as they form collaterals from the lateral funiculus (Perrin *et al.*, 2001).

We used in ovo RNAi to perturb the expression of SynCAM1, SynCAM2 and SynCAM3 in the DRGs. We first analyzed the development and pathfinding of sensory axons in whole-mount preparations of HH24.5/HH25 embryos stained with an anti-neurofilament antibody (Fig. 11A-F). Loss of all three SynCAMs individually resulted in abnormal entry of sensory afferents into the dorsal spinal cord (Fig. 11A-E). Seventy-three percent of the embryos injected with GFP and 88% of the untreated control embryos showed a homogenous dorsal longitudinal sensory axon bundle which was of constant thickness along the anterior-posterior (AP) axis. However, in 25% of embryos injected with dsRNA against SynCAM1, 37% of embryos injected with dsSynCAM2 and 36% of embryos injected with dsSynCAM3 the longitudinal axon bundle was variable in thickness with a wavy appearance. In contrast, only 8% of GFP-injected and untreated control embryos showed this abnormal phenotype. When we measured the relative thickness of the longitudinal axon bundle in experimental and

control embryos we found a significantly reduced ratio of bundle size in the region between two DRGs and bundle thickness at the root entry (Fig. 11F).

To find an explanation for the aberrant morphology of the longitudinal axon bundle, we looked at the DREZ in cross-sections of the spinal cord (Fig. 11G-I). Spinal cord sections collected from HH25 experimental and control embryos were stained with an anti-axonin1 antibody to visualize the sensory axon tract. Knockdown of SynCAM2 and SynCAM3 resulted in segmentation of the axon bundle. Instead of the smooth oval shape and the regular arrangement of axons seen in control embryos, we found gaps and morphological changes in 33% of embryos with reduced levels of SynCAM2 and in 38% of embryos with reduced levels of SynCAM3 (Fig. 11J). Downregulation of SynCAM1 had no effect. Segmented sensory axon bundles were observed in 19% of embryos injected with dsSynCAM1. In contrast, only 11% of embryos injected with GFP and 13% of untreated control embryos exhibited segmentation of the sensory axon bundle.

In summary, knockdown of SynCAMs resulted in an inhomogeneous sensory axon bundle with variable thickness and segmentation. These findings are in line with the in vitro data suggesting that SynCAMs play an important role in regulating axon-axon contact and selective fasciculation of sensory axons, which in turn could lead to correct pathfinding of sensory afferents in the dorsal spinal cord.

Discussion

Until a few years ago SynCAMs were only implicated in late steps of neural circuit formation including synaptogenesis and myelination. However, recently these molecules were shown to be necessary in the guidance of post-crossing commissural axons (Niederkofler *et al.*, 2010). In line with these findings, our study supports a role of SynCAMs in axon guidance as we could show an involvement of these molecules in proper entry and pathfinding of sensory afferents along the longitudinal axis of the dorsal chicken spinal cord in vivo. We provide strong evidence for the mechanism underlying sensory axon pathfinding based on our finding that selective axon-axon interaction is perturbed in the absence of SynCAM family members. SynCAMs could control selective contacts between axons via their complex interaction pattern, including homo- and heterophilic cis- as well as trans-interactions, which

could elicit different intracellular responses necessary for the correct interpretation of external guidance cues.

Complex cis-/trans-interaction pattern between SynCAMs could elicit different intracellular signaling

Prior to interactions in trans, SynCAMs assemble in cis to form dimers or oligomers (Fogel *et al.*, 2011). So far, only homophilic cis-complexes have been reported whereas trans-interactions were shown to be homo- or heterophilic. Heterophilic adhesion is in generally stronger than homophilic interactions (Fogel *et al.*, 2007; Maurel *et al.*, 2007; Niederkofler *et al.*, 2010; Thomas *et al.*, 2008). In our hands we were able to reproduce these data. We used purified SynCAM proteins to test their binding properties in vitro. Interestingly, in addition to homophilic cis-assembly, we found the formation of a heterophilic cis-complex between SynCAM1 and SynCAM2, which was able to weaken trans-binding of SynCAMs.

SynCAM1 and SynCAM2 were found to be required for the guidance of post-crossing commissural axons (Niederkofler *et al.*, 2010). After perturbation of axonally expressed SynCAM1 and SynCAM2 as well as floor-plate-derived SynCAM2 commissural axons crossed the floor plate but failed to turn in the rostral direction. While the strong heterophilic interaction between axonal SynCAM1 and floor-plate SynCAM2 accounts for these pathfinding errors at the midline, the weak homophilic interaction of SynCAM2 cannot explain the erroneous navigation after its knockdown in commissural axons. In agreement with our findings, axonally expressed SynCAM2 could form a heterophilic cis-complex with coexpressed SynCAM1, thereby modulating the strong adhesion to SynCAM2 in the floor plate, consequently facilitating the turning response into the longitudinal axis.

From studies at the synapse and non-neuronal cells it is known that SynCAMs associate with different intracellular binding partners including proteins of the MAGUK family, such as CASK, Dlg3 and Pals2, members of the protein 4.1 family and the guanine nucleotide exchange factor Farp1 (Cheadle & Biederer, 2012; Hoy *et al.*, 2009; Kakunaga *et al.*, 2005; Shingai *et al.*, 2003; Yageta *et al.*, 2002; Zhou *et al.*, 2005). The complex cis-/trans-interaction pattern and the resulting SynCAM complexes might recruit different intracellular effector molecules, thereby eliciting specific responses and, thus, fine-tune the behavior of axons and their growth cones (Fig. 12).

SynCAMs act as recognition molecules regulating selective contact and fasciculation between sensory axons

Based on our model specific intracellular SynCAM-derived signaling is dependent on the formation of different interaction complexes. Changing the levels of SynCAM expression would therefore alter the composition of SynCAM complexes, which in turn could result in changes of the behavior of axons and growth cones. Indeed, our results support this hypothesis. Both, adding SynCAM externally as a substrate and perturbing endogenously expressed SynCAM in DRGs resulted in decreased axon-axon contact and, as a result, increased branching of axons or filopodia from main bundles. In this scenario, SynCAMs offered as substrate would act as dominant negative effectors on axon-axon interactions. As a consequence of this the sensory neural network was disorganized, with less stable axon fascicles, frequent axonal crossing between bundles and no orientation of growth.

A role of different SynCAM interaction complexes in regulating selective fasciculation is in agreement with the function of other members of the IgCAM-superfamily. In several studies it has been shown that the formation of an axonin1-NgCAM cis-cluster determines the behavior of axons. Besides promoting outgrowth of sensory neurites by interacting with NgCAM as a substrate (Buchstaller *et al.*, 1996; Stoeckli *et al.*, 1996), this complex regulates fasciculation of sensory axons (Kunz *et al.*, 1996; Kunz *et al.*, 1998). Appearing mainly in their monomeric form on isolated sensory neurons, with increasing neurite fasciculation axonin1 and NgCAM cluster to form heterodimeric and heterotetrameric complexes. Thereby intracellular signaling is changed with a decrease in axonin1-associated fyn kinase activity and concomitant increase in NgCAM-associated casein kinase II-related activity. In turn, these changes were suggested to stabilize neurite-neurite contacts (Kunz *et al.*, 1996). Taken together, this shows that through the formation of different cis-/trans-complexes and the resulting intracellular signaling, IgSF-CAMs act as recognition molecules on the surface of axons and growth cones, which contribute to the pathfinding of axons by selective fasciculation (Fig. 12). The fact that IgSF-CAMs such as axonin1 or NgCAM interact with many other cell adhesion molecules including NrCAM, F11 and NCAM (Brummendorf *et al.*, 1993; Milev *et al.*, 1996; Morales *et al.*, 1993; Perrin *et al.*, 2001; Stoeckli & Landmesser, 1995; Stoeckli *et al.*, 1997; Stoeckli *et al.*, 1996; Suter *et al.*, 1995) prompts speculations about the potential interplay between SynCAM family members and those other IgSF-CAMs.

Changes in growth cone morphology and SynCAM expression suggest an active contribution of SynCAMs to the signal transduction of guidance cues

In line with a role of SynCAMs as recognition molecules on axon and/or growth cone surfaces we observed changes in growth cone morphology and distribution of SynCAM molecules during contact with SynCAMs presented as a substrate. Growth cones grown on SynCAM substrate were much larger than those on laminin, but had fewer filopodia per area. An enlargement in growth cone size has been linked to preference responses (Fitzli *et al.*, 2000). In that study the authors showed that commissural axons grown on alternating stripes of NgCAM/NrCAM and NgCAM displayed enlarged growth cones. Alternating stripes of NrCAM and NgCAM did not elicit a choice reaction and under these conditions, growth cones remained small. This shows that the change in substrate and the corresponding preference response elicited the increase in growth cone size. However, in our study, the substrate contained SynCAM molecules of one kind only and thus, the increase in growth cone size cannot be caused due to a choice response.

In addition to an enlargement of the growth cone area, the distribution of membrane expressed SynCAMs changed upon contact with SynCAM substrates. SynCAM1 and SynCAM2 were redistributed to the basal surface of the growth cone when contacting either SynCAM1 or SynCAM2 substrate but not laminin. This suggests that SynCAMs are actively recruited to sites of contact where they probably transduce signals elicited by external guidance cues, in this case SynCAMs, to the cytoskeleton of the growth cone. The driving force for the movement of SynCAM molecules to the substrate-facing surface could be the strong heterophilic interaction between SynCAM1 and SynCAM2. However, we also observed a redistribution of SynCAM1 on SynCAM1 substrate and of SynCAM2 on SynCAM2 substrate. In agreement with others, our data reveals that homophilic interactions are weak (Fogel *et al.*, 2007) or even undetectable (Niederkofler *et al.*, 2010). Therefore, it is highly unlikely that a weak homophilic interaction would be strong enough to relocate SynCAM molecules to the basal membrane. Alternatively, in case of a SynCAM1-SynCAM2 heterophilic cis-assembly, SynCAM1 would be recruited due to the strong heterophilic trans-interaction of SynCAM2 with substrate SynCAM1. Due to the finding that binding of SynCAM1 and SynCAM2 ectodomains to SynCAM1/SyCAM2 co-transfected cells was strongly reduced, it is not obvious that the heterophilic cis-complex would be relocated to the substrate-facing

membrane by an interaction with SynCAM partners in trans. However, reduction in binding strength cannot be correlated to reduction in signaling. Depending on the context, the response elicited by an interaction of the hetero-cis-complex with SynCAMs in trans might be different. In this case, the response elicited when axons and growth cones were in contact with SynCAM substrates was specific as seen by the change in growth cone morphology compared to control substrates.

Similar findings were obtained with axonin1 and NgCAM. When presented as a substrate growth cones on axonin1 substrate were on average twice as large as those on NgCAM or laminin (Stoeckli *et al.*, 1996). Furthermore, on axonin1 or NgCAM substrate, membrane-expressed axonin1 and NgCAM accumulated at the substrate-facing membrane of growth cones. On NgCAM substrate, axonin1 was cleared form the surface area as well, although not completely, mainly remaining at the front of the lameliopodia and on filopodia of the growth cone. In this case, the major driving forces were thought to be homophilic NgCAM trans-interactions, homophilic axonin1 trans-interactions as well as heterophilic cis-assembly of axonin1 and NgCAM. Together, these data indicate that the growth cone responds to different substrates with a specific morphology and specific distribution of SynCAMs, axonin1 and NgCAM on its surface. Thus, these molecules might actively contribute to signal transduction and responses of axons and growth cones to guidance cues.

The function of SynCAMs in early neural circuit formation is supported by their involvement in sensory axon pathfinding in vivo

The involvement of SynCAMs in the regulation of selective axon-axon contacts suggested a role of these molecules in sensory axon pathfinding. A direct contribution of SynCAMs in pathway selection was supported by our in vivo findings showing that knockdown of SynCAMs induced aberrant pathfinding of sensory afferents in the dorsal spinal cord of the chicken embryo. As hypothesized above, altering the expression of one SynCAM family member can change the interaction profile and thus, alter selective axon-axon contact and fasciculation. In turn, disrupted selective fasciculation would result in improper bundle formation, which could be a cause for the inhomogeneous thickness and the segmentation of the sensory axon bundle along the longitudinal axis (Fig. 13). The wavy appearance of the longitudinal bundle cannot be explained by a lack of growth since we showed that the

contribution of SynCAMs to axon elongation is minor at these early stages of sensory axon pathfinding. This is in contrast to a vast majority of IgSF-CAMs, including axonin1, NgCAM, NrCAM and F11, which have been found to promote sensory axon elongation early on in development (Buchstaller *et al.*, 1996; Kuhn *et al.*, 1991; Lustig *et al.*, 1999; Morales *et al.*, 1993; Stoeckli *et al.*, 1991; Stoeckli *et al.*, 1996). At the same time our results show that IgSF-CAMs can have very different functions. Interestingly, SynCAMs seem to be involved in the promotion of sensory axon outgrowth at later stages. Hence, SynCAMs exert different functions at different time points of neural circuit formation even before synaptogenesis and myelination. In addition to this, we found SynCAMs expressed in a subtype-specific profile in older DRGs (HH30-34), SynCAM1 being present in nociceptive neurons and SynCAM2 expressed in proprioceptive neurons. This suggests that SynCAMs might be required for axon elongation and possibly also pathfinding of sensory collaterals as well as synapse formation with their targets in the grey matter of the spinal cord. A function of SynCAMs in early sensory axon guidance and a potential role in sensory collateral pathfinding is in line with the roles of other IgSF-CAMs. Axonin1 and F11 have been found to be important for sensory axon guidance into and along the longitudinal axis of the spinal cord (Perrin *et al.*, 2001). Later, the differential interactions between axonin1 and NgCAM as well as F11 and NrCAM are necessary for subpopulation-specific pathfinding of sensory collaterals to targets in the grey matter. Axonin1 and NgCAM were shown to be involved in nociceptive and F11 and NrCAM in proprioceptive collateral guidance (Perrin *et al.*, 2001). Thus, SynCAMs together with other IgCAMs might be responsible for proper sensory neural circuit formation. Whether they act in a common pathway or in an additive fashion needs to be investigated in the future.

Etiologies of neurodevelopmental diseases go beyond defective synapses

Our study demonstrates the importance of SynCAMs throughout the development of neural circuits. Originally discovered as synaptic cell adhesion molecules, SynCAMs have been shown to play important roles in commissural as well as sensory axon pathfinding, processes occurring prior to synaptogenesis. Lately, two missense mutations in the gene encoding for SynCAM1 have been found in patients diagnosed with autism spectrum disorder (ASD) (Zhiling *et al.*, 2008). So far neurodevelopmental disorders such as ASD have been linked to defective synaptogenesis and deficits in synaptic plasticity. However, our results demonstrate that SynCAMs are important early on in neural circuit formation and therefore suggest that the underlying pathology of neurodevelopmental diseases could involve more neural circuit deficits than just aberrant synapse formation and plasticity. Indeed, changes in axonal connectivity have been associated with autism (Geschwind & Levitt, 2007) supporting the idea that disrupted axonal pathfinding contributes to the etiology of neurodevelopmental diseases.

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Tables

Table 1. Values of the total axon length and the longest axon per neuron of E5 sensory neurons, 48 hours in culture

Total axon length (average)				Longest axon per neuron (average)		
E5	0.4µg/ml	10µg/ml	50µg/ml	0.4µg/ml	10µg/ml	50µg/ml
SynCAM1	237.4	223.9	249.5	180.7	185.0	216.3
SynCAM2	244.4	242.8	253.0	203.4	198.0	212.4
SynCAM3	210.9	252.5	222.9	165.9	215.3	200.5
Albumax	186.0	184.3	185.2	148.4	144.1	141.7
PLL	189.0	189.0	189.0	141.9	141.9	141.9

Table 2. Values of the total axon length and the longest axon per neuron of E8 sensory neurons, 48 hours in culture

Total axon length (average)				Longest axon per neuron (average)		
E8	0.4µg/ml	10µg/ml	50µg/ml	0.4µg/ml	10µg/ml	50µg/ml
SynCAM1	280.5	302.0	260.6	223.3	245.5	226.8
SynCAM2	306.8	312.6	450.8	266.5	270.0	367.4
SynCAM3	300.4	404.2	512.1	239.5	315.4	412.3
Albumax	197.1	200.3	189.2	164.7	168.1	153.2
PLL	200.0	200.0	200.0	166.0	166.0	166.0

Table 3. Values of the total axon length and the longest axon per neuron of E8 sensory neurons, 28 hours in culture

Total axon length (average)				Longest axon per neuron (average)		
E8	0.4µg/ml	10µg/ml	50µg/ml	0.4µg/ml	10µg/ml	50µg/ml
SynCAM1	101.8	115.6	126.9	84.8	95.5	105.8
SynCAM2	118.8	134.4	132.8	102.2	110.2	112.8
SynCAM3	104.8	101,.7	119.9	84.6	87.2	98.9
Albumax	85.1	75.6	72.5	67.7	61.0	54.9
PLL	74.1	74.1	74.1	62.7	62.7	62.7

Figure legends

Figure 1

Purity of SynCAM ectodomains. Comparison of silver-stained gels and Western blots after SDS-PAGE show that the ectodomains of chicken SynCAM1, SynCAM2 and human SynCAM3 were pure. (A-C) Ectodomains used for the binding studies were tagged with an AP-myc-6xHis-tag. (D-F) Ectodomains used as antigens and for coating of surfaces were fused to a 6xHis-tag only. (A-F) The amount of protein loaded is indicated on top of each lane.

Figure 2

Specificity of SynCAM antibodies. (A-C) Anti-SynCAM1 (A), anti-SynCAM2 (B) and anti-SynCAM3 (C) antibodies do not crossreact with other SynCAM family members. SDS-gels were loaded with HEK293T cell lysates transfected with full-length SynCAM1 (lane 2), SynCAM2 (lane 3), human SynCAM3 (lane 1) and with lysates of untransfected cells (lane 4). Western blots were stained against anti-SynCAM1 (A), anti-SynCAM2 (B) and anti-SynCAM3 (C). The antibodies only recognized the corresponding SynCAM family member. SynCAMs are not endogenously expressed in HEK293T cells (lane 4). (D-F) SDS-gels for Western blots were loaded with lysates of E5 chicken spinal cord (SC) (E and lane1 in D, F), E5 DRGs (lane 2 in D and F), E8 DRGs (lane3 in D and F) and lysates of HEK293T cells transfected with full-length chicken SynCAM1 (D, lane 4) and human SynCAM3 (F, lane4). (D) Anti-SynCAM1 antibody detected endogenous SynCAM1 in spinal cord (lane1) as well as in DRG lysates (lane 2 and 3) at the expected size of around 100 kDa (Fogel *et al.*, 2007). SynCAM1 overexpressed in HEK293T cells runs at a slightly lower molecular weight, around 70 kDa (lane 4). Note the pattern of multiple bands, which most probably represents differently glycosylated forms. As a negative control, Western blots were incubated with the preimmune serum (PI) of the same rabbit. (E) Staining of E5 chicken spinal cord lysate with anti-SynCAM2 antibody revealed multiple weak bands, two prominent bands between 100 and 130 kDa and one above 55 kDa. Multiple weak bands were also recognized when blots were incubated with the preimmune serum (PI) of the same rabbit. (F) The anti-SynCAM3 antibody detected multiple bands with one prominent band at 70 kDa in Western blots of all chicken tissue lysates (lane 1 to 3). The antibody specifically recognized human full-length SynCAM3 overexpressed in HEK293T cells (lane 4). Many background bands were visible on blots stained with the preimmune serum (PI) of the same rabbit.

Figure 3

Efficiency and specificity of SynCAM knockdown. (A-L) Efficiency and specificity of SynCAM knockdown was tested by transfecting HEK293T cells with destabilized GFP fused to either SynCAM1-3'UTR (A-D), SynCAM2-3'UTR (E-H) or SynCAM3-3'UTR (I-L) together with siRNAs against different SynCAM family members and a tomato fluorescent protein construct. SynCAM1 was efficiently downregulated by siSynCAM1 (B) but not by siSynCAM2 (C) or siSynCAM3 (D). The same was true for SynCAM2, which was only knocked down by siSynCAM2 (G) but not by siSynCAM1 (F) or siSynCAM3 (H). The expression of SynCAM3 was effectively decreased after co-transfecting siSynCAM3 (L) but not after co-transfecting siSynCAM1 (J) or siSynCAM2 (K). The transfection rate was high and comparable among the different conditions visualized by the expression of tomato fluorescent protein. Expression of the destabilized GFP-SynCAM-3'UTR constructs (A, E, I) without transfection of siRNA was comparable to the expression of these constructs with transfection of the non-targeting siRNAs. SiRNAs were produced by digestion of the long dsRNAs used in the in vivo experiments. (M) Quantification shows a significant knockdown of SynCAM1 by siSynCAM1, of SynCAM2 by siSynCAM2 and of SynCAM3 by siSynCAM3 compared to SynCAM expression in the absence of siRNAs. Two asterisks indicate a p-value<0.01 and three asterisks indicate a p-value<0.001 for the comparison of the signal intensities of destabilized GFP-SynCAM constructs with and without siRNA. (N, O) Efficiency of SynCAM1 knockdown in vivo in the chicken spinal cord. (N) Lysates of commissural neurons of the electroporated (e) and the control (c) half of the spinal cords of different embryos (#1-#5) were loaded on an SDS-gel, blotted and stained for endogenous levels of SynCAM1 using anti-SynCAM1 antibody. As a control, lysates of untreated embryos were analyzed (#6, #7). Similar levels of SynCAM1 expression were found on both sides of the spinal cord (L=left side, R=right side). (O) Quantification of signal intensities shows a significant reduction of SynCAM1 protein on the electroporated side versus the control side. Quantification includes measurements from lysates of commissural and motoneurons. SynCAM1-signals were normalized to GAPDH-signals. One asterisk indicates a p-value<0.05 for the comparison of SynCAM expression on electroporated versus control sides. (M, O) Statistics were done with the two-tailed Student's t-test. Values are given as mean +/- s.e.m.

Figure 4

SynCAM1 and SynCAM2 strongly interact in a heterophilic manner.

(A-F) HeLa cells transfected with SynCAM1-HA (A, B), SynCAM2-HA (C, D) and empty vector (E, F) were incubated with purified myc-tagged SynCAM1 (A, C, E) or SynCAM2 ectodomains (B, D, F). Homophilic binding of SynCAM1^{ecto} to SynCAM1 (A) and SynCAM2^{ecto} to SynCAM2 (D) was weak. SynCAM2^{ecto} (B) and SynCAM1^{ecto} (C) strongly bound to SynCAM1- (B) and SynCAM2-transfected cells (C), respectively. SynCAM1^{ecto} (E) and SynCAM2^{ecto} (F) did not bind to mock-transfected HeLa cells. Insets show higher magnifications of cells with bound ectodomains. Binding of ectodomains was visualized using an anti-myc antibody (red) and transfection of cells with full-length SynCAMs was visualized using an anti-HA antibody (green). Images were not taken with the same exposure times. Detection of weak homophilic binding was only possible when longer exposure times were used.

(G-I) Co-immunoprecipitation of homophilic and heterophilic SynCAM interactions. For the immunoprecipitation (IP) anti-myc- (G), anti-Flag- (H) and anti-HA-coupled agarose beads (I) were used. Beads were incubated with lysates of HEK293T cells co-transfected with full-length SynCAMs or empty vector. SynCAM1-HA was pulled down with SynCAM1-myc (G, lane 4), SynCAM2-HA with SynCAM2-Flag (H, lane 4) and SynCAM2-Flag with SynCAM1-HA (I, lane 4). Homophilic interaction between SynCAM2 was only weakly detectable (H, lane 4). Input lysates of HEK293T cells show the successful co-transfection of SynCAMs (lane 2). Anti-myc, anti-HA and anti-Flag antibodies did not stain any unspecific bands in the mock input lysate (lane 1). Also, mock transfected cells did not pull down any unspecific proteins (lane 3).

Figure 5

Heterophilic cis-complexes between SynCAM1 and SynCAM2 modify binding in trans.

(A-E) Reduction in binding of SynCAM ectodomains to SynCAM1/SynCAM2 co-transfected HeLa cells. Binding of SynCAM1^{ecto} (C) and SynCAM2^{ecto} (D) to co-transfected cells was significantly reduced compared to heterophilic binding between SynCAM1^{ecto} and SynCAM2-expressing cells (A) and SynCAM2^{ecto} and SynCAM1-expressing cells (B). Homophilic SynCAM1 and homophilic SynCAM2 interactions are not depicted. Bound ectodomains were visualized using an anti-myc antibody (red), transfected full-length SynCAMs were visualized using anti-Flag (green) or anti-HA antibody (blue). (E) Quantification of the binding of SynCAM1 and SynCAM2 ectodomains to single- (homophilic binding depicted in white, heterophilic binding depicted in dark grey) and co-transfected cells (light grey). Note that binding of SynCAM2^{ecto} to co-transfected cells is more reduced than binding of SynCAM1^{ecto} to co-transfected cells. For the quantification of the binding strength pictures were taken with constant settings (exposure time, upper/lower limit). Values of one out of seven representing experiments are depicted. Two asterisks indicate a p-value<0.01 for the comparison of each condition to heterophilic binding using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m.

(F-M) Reduction in ectodomain binding is not an artefact due to the unavailability of binding partners on cell membranes. Cells were separately transfected with either SynCAM2-Flag (H, green) or SynCAM1-HA (I, blue) or with SynCAM1-Flag (L, green) or SynCAM2-HA (M, blue). Cells were mixed (G, K) and incubated with SynCAM ectodomains (F, J, red). SynCAM1^{ecto} (F, open arrowheads indicate absence of SynCAM1^{ecto}) was not able to compete with heterophilic cell-cell contacts between SynCAM1-HA and SynCAM2-Flag (G, open arrowheads). However, there was still SynCAM2-Flag on the cell membrane, which was not involved in the contact site and, thus, was available for SynCAM1^{ecto} binding (F and G, white arrowheads). The same was true for SynCAM2^{ecto} (J, open arrowheads), which was not able to bind to SynCAM1-Flag-SynCAM2-HA cell-cell contacts (K, open arrowheads). However, SynCAM1-Flag was still available on cell membranes to interact with SynCAM2^{ecto} (J and K, white arrowheads).

(N, O) Cross-linking of SynCAMs in the plane of the cell membrane reveals heterophilic cis-interactions between SynCAM1 and SynCAM2. HEK293T cells were co-transfected with either SynCAM1-HA and SynCAM1-Flag (positive control) or SynCAM1-HA and SynCAM2-Flag. Cells were isolated and incubated with the cross-linker BS^3. Cell lysates were submitted to co-immunoprecipitation. Western blots were stained with anti-HA- (N) or anti-Flag-antibodies (O). Lane 1 to lane 4 represents the input lysates showing successful co-transfection of SynCAM1-HA (N, lane1)

and SynCAM1-Flag (O, lane 1) as well as SynCAM1-HA (N, lane 2) and SynCAM2-Flag (O, lane 2). Input lysates were loaded as undiluted (N, O, lane 1 and 2) and 1 to 10 diluted (N, O, lane 3 and 4) samples. (N) Anti-HA staining shows that SynCAM1-HA was pulled down using anti-HA-antibody-coupled agarose beads both in the monomeric form (arrowheads) and as multimeric complexes (black arrowhead) when BS^3 was added (lane 5 and 6). In the absence of the cross-linker, SynCAM1-HA was only present as monomer (lane 7 and 8). The band at 130 kDa (asterisk) could not be clearly identified and could represent either an unspecific band or SynCAM1 dimers. This band was not detected on Western blots loaded with lysates of HEK293T cells overexpressing SynCAM1 and stained with anti-SynCAM1 antibody (see Fig. 2D, lane 4). (O) Anti-flag staining revealed the presence of SynCAM1-Flag (lane 5) and SynCAM2-Flag (lane 6) in the high molecular weight complexes (black arrowhead). Without cross-linker the high molecular weight complexes were not detected and SynCAM1- (lane 7) and SynCAM2-Flag (lane 8) were only present as monomers (arrowheads). GAPDH served as loading control.

Figure 6

SynCAMs are expressed in DRG sensory neurons, axons and growth cones during the time of sensory axon pathfinding.

The expression pattern of SynCAM family members was analyzed by in situ hybridization (A-U) and immunostaining (V-Z) on chicken (A-W) and mouse (X) spinal cord cross sections as well as on dissociated chicken sensory neurons (Y, Z).

(A-R) At stage HH11 and HH14, SynCAM1 was present in somites (A, D, black arrows). By HH18 SynCAM1 mRNA was also detectable in the floor plate (arrowhead), in motoneurons (arrow), in DRGs (black arrow) and in the dermomyotome (asterisk) (G). SynCAM2 expression only started at HH18 (H), no signal was detectable at HH11 (B) and HH14 (E). At HH18, SynCAM2 was expressed in motoneurons (arrow), the floor plate (arrowhead), weakly in DRGs (black arrow) and in the dermomyotome (asterisk) (H). In contrast, SynCAM3 expression in the floor plate was found already at HH11 (C, arrowhead). Expression of SynCAM3 was unchanged at HH14 (F). At HH18, SynCAM3 was still expressed in the floor plate (arrowhead) but now also started to be detectable in motoneurons (arrow) and in the roof plate (asterisk) (I). Expression of all SynCAMs was similar by HH21. SynCAM1 (J) was found in the floor plate (arrowhead), in motoneurons (arrows), and in DRGs (black arrowhead). The same was found for SynCAM2 (K). SynCAM3 (L) was found in the floor plate (arrowhead) at even higher levels than before and in the DRGs (black arrow). At HH23, SynCAM1 (M) was no longer found in the floor plate, in contrast to SynCAM2 (N, arrowhead) and SynCAM3 (O, arrowhead). In addition to previous stages, SynCAMs were now also expressed in commissural neurons in the dorsal spinal cord (M-O, white arrowheads). At HH26, the expression pattern of SynCAM1 (P), SynCAM2 (Q), and SynCAM3 (R) were largely unchanged. (S-U) At HH30 and HH34, SynCAM1 (S) and SynCAM2 (T) expression was restricted to the dorsomedial and the ventrolateral region of the DRG, respectively. Insets depict staining for TrkA (S) and TrkC (T). Note that SynCAM1 and TrkA as well as SynCAM2 and TrkC stain similar regions in the DRG. SynCAM3 showed a more widespread expression in stage HH30 and HH34 DRGs with increasing signal at HH34 (U).

(V-X) Immunostaining of SynCAM1 (V) and SynCAM2 (W) on chicken HH26 spinal cord cross sections revealed an expression in the dorsomedial and ventrolateral DRG (arrows), respectively, in the dorsal sensory axon tract (grey arrowheads) and in the commissure and post-crossing commissural axons in the ventral spinal cord (white arrowheads). SynCAM2 was additionally detectable in the FP at that stage (W, arrowhead). The antibody against human SynCAM3 recognized SynCAM3 expression in DRGs (arrow) and sensory axons (grey arrowhead) in mouse spinal cord cross sections (X). (Y, Z) SynCAM1 (Y) and SynCAM2 (Z) were present on the surface of axons and growth cones of dissociated

sensory neurons. Note the strong staining in filopodia (arrows). Inset in (Z) shows axonin1 immunoreactivity visualizing the axon and growth cone. Staining was done on unfixed neurons to detect surface-expressed proteins only.

Figure 7

SynCAMs provide an adhesive substrate for sensory axons.

(A-D) Eight-day-old sensory neurons were cultured on COS7 cells expressing HA-tagged SynCAM1 (A), SynCAM2 (B), SynCAM3 (C) and MARCKS-GFP (mGFP) (D). Axons were visualized by anti-neurofilament staining (red), transfected SynCAMs by anti-HA staining (green). White arrowheads mark growth cones ending on transfected cells and open arrowheads mark growth cones ending on untransfected cells. (E, F) Calculation of the ratio between growth cones ending on transfected cells (white arrowheads in A-D) and the total number of growth cones (all arrowheads in A-D) of sensory axons dissected from E5 (E) and E8 embryos (F). This ratio was normalized to the transfected area (green). For both E5 (E) and E8 axons (F), significantly more growth cones stopped on cells expressing SynCAM1, SynCAM2 and SynCAM3 compared to cells expressing mGFP. Two asterisks indicate a p-value<0.01 for the comparison between mGFP and all other groups using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m.

Figure 8

SynCAMs promote neurite outgrowth of E8 but not of E5 sensory neurons.

Dissociated sensory neurons of E5 (A-G) and E8 embryos (H-U) were cultured on substrates containing 50 µg/ml (A, B, H, I, O, P), 10 µg/ml (C, D, J, K, Q, R) or 0.4 µg/ml (E, F, L, M, S, T) SynCAM1, SynCAM2 or SynCAM3 ectodomains or Albumax. All dishes were precoated with 10 µg/ml poly-L-lysine (PLL). Axons were grown in culture for 48 hours (A-M) or for 28 hours (O-U). Neurite lengths were measured and the total axon length per neuron (A, C, E, H, J, L, O, Q, S), the longest axon per neuron (B, D, F, I, K, M, P, R, T) and the substrate-concentration dependence on the growth response (G, N, U) were quantified. (A-G) E5 sensory axons showed significantly increased values for the total axon length and the longest axon per neuron when grown on 50 µg/ml SynCAM2 substrate (A, B). Only the longest neurite but not the total axon length was longer on SynCAM1 substrate (B) compared to the values of axons grown on PLL. SynCAMs coated at a concentration of 10 µg/ml (C, D) and 0.4 µg/ml (E, F) did not significantly promote axon length. (G) For any SynCAM E5 sensory axons did not respond to different concentrations and were of similar length under all conditions. (H-N) For sensory neurons dissected from E8 embryos, both the total axon length (H, J, L) as well as the length of the longest axon per neuron (I, K, M) were significantly longer when grown on 50 µg/ml (H, I), 10 µg/ml (J, K) and 0.4 µg/ml (L, M) SynCAM2 or SynCAM3 substrate. On 10 µg/ml of SynCAM1 substrate the total axon length (J) and the longest axon per neuron (K) were significantly increased whereas on 0.4 µg/ml SynCAM1 substrate (L, M) only the total axon length was significantly longer compared to PLL (L). (N) Concomitantly with increasing concentrations of SynCAM2 and SynCAM3 also the total axon length increased. In contrast, axons on SynCAM1 substrate were of similar length independent of the concentration. (O-U) E8 axons grown for 28 hours in culture showed significantly increased total axon length (O, Q, S) and length of the longest axon per neuron (P, R, T) on all concentrations of coated SynCAM substrate. (U) The outgrowth promoting effect after 28 hours in culture was independent of the substrate concentration. Statistics was done using ANOVA/Tukey HSD post-hoc test. One asterisk indicates a p-value<0.05 and two asterisks indicate a p-value<0.01 for the comparison between each condition and PLL. Values are given as mean +/- s.e.m. The absolute values of each experiment are depicted in table 1-3.

Figure 9

SynCAMs influence selective axon-axon contacts.

(A-F) Scanning electron micrographs of single axons grown from intact DRGs cultured on SynCAM1 (A), SynCAM2 (B) and SynCAM3 ectodomains (C), laminin (D), Albumax (E) and PLL (F). On SynCAM substrate (A-C) more filopodia (arrowheads) branch off a neurite compared to the control substrates (D-F). (G, H) Quantification reveals a significant increase in the number of filopodia per µm of neurite (G) and a significant higher percentage of filopodia with higher order branches (H) when neurites were grown on SynCAM substrate compared to control substrates. One asterisk indicates a p-value<0.05 and two asterisks a p-value<0.01 for the comparison between of SynCAM1, SynCAM2 and SynCAM3 to PLL (G) and Albumax (H) using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m.

(I-S) Scanning electron micrographs taken from the peripheral axonal network of intact DRGs cultured on SynCAM1 (I), SynCAM2 (J) and SynCAM3 ectodomains (K), laminin (L), Albumax (M), PLL (N) or collagen (O-S). (I-N) SynCAM1 (I), SynCAM2 (J) and SynCAM3 (K) used as substrate caused a disorganization of the axonal network with more axons branching off main bundles and a concomitant crossing of axons between bundles. On laminin (L), Albumax (M) and PLL (N), axons changed bundles at lower frequency resulting in a more organized, radial outgrowth pattern. (O-S) DRGs injected with dsRNA against SynCAM1 (O), SynCAM2 (P) or SynCAM3 (Q) together with a GFP plasmid, GFP-control injected DRGs (R) and untreated control DRGs (S) were cultured on collagen. Compared to the control conditions (R, S), knockdown of SynCAMs (O-Q) resulted in changed a morphology of the neural network with less defined bundles and an increase in axons crossing between bundles.

Figure 10

SynCAMs affect growth cone morphology and their distribution on the growth cone surface.

(A-I) Scanning electron micrographs of growth cones of intact DRGs cultured on SynCAM1 (A), SynCAM2 (B) and SynCAM3 ectodomains (C) and laminin (D), Albumax (E) and PLL (F). Growth cones were markedly enlarged on SynCAM substrates (A-C) compared to growth cones on control substrates (D-F). (G) Quantification of the growth cone area shows a significant increase in size on SynCAM1, SynCAM2 and SynCAM3 substrate compared to the control substrates. (H) Quantification of the number of filopodia normalized to the growth cone area. This number was significantly reduced when growth cones were cultured on SynCAM2 substrate. One asterisk indicates a p-value<0.05 and two asterisks indicate a p-value<0.01 for the comparison between SynCAMs and the control substrate PLL (G, H) and between SynCAM1 and the control substrate Albumax (G) using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m. (I) Quantification of the different growth cone shapes in dependence of the substrate. On SynCAM2 growth cones were primarily round or long and flat whereas on SynCAM1, SynCAM3 and PLL growth cones had a round or finger-like morphology. On laminin and Albumax growth cones mainly displayed a finger-like shape and to a lesser extent a roundish morphology. Long and thin growth cones were mostly found on PLL, Albumax, laminin and SynCAM1, but never on SynCAM2 or SynCAM3 substrates.

(J-O) SynCAM1 (J, J', K, K') and SynCAM2 (M, M', N, N') were redistributed to the substrate-facing surface of the growth cone when grown on SynCAM1 (J, J', N, N') and SynCAM2 substrate (K, K', M, M'), respectively. On laminin, SynCAM1 (L, L') and SynCAM2 (O, O') were present on the apical surface. Note that on SynCAM2 (K, K') and SynCAM1 (N, N') substrate, SynCAM1 and SynCAM2 are detectable at the outer rim and the filopodia of the growth cones. Insets in (M, M'), (N, N') and (O, O') show growth cones stained for axonin1. Staining was done on unfixed neurons to detect surface-expressed proteins only.

Figure 11

SynCAMs are required for proper pathfinding of sensory afferents.

(A-D) Whole-mount preparations of HH24.5/HH25 chicken embryos stained for neurofilament. Downregulation of SynCAM1 (A), SynCAM2 (B) and SynCAM3 (C) using dsRNA caused an abnormal entry of sensory afferents into the dorsal spinal cord. The sensory bundle was thicker in the region of the dorsal root entry (white arrows) compared to the region between DRGs (open arrows). The sensory axon bundles of control embryos (D) were of the same thickness along the AP-axis (white and open arrows). Overview picture depicts the lumbosacral region of a whole-mount embryo. Box marks the area of the spinal cord and the DRGs magnified in (A-D). (E) Quantification of the number of embryos showing abnormal entry of sensory afferents into the dorsal spinal cord. Significantly more abnormal phenotypes were observed after knockdown of SynCAM2 and SynCAM3. After downregulation of SynCAM1 we also found an increase of abnormal phenotypes but the value was not significantly different from controls. Depicted values represent percentages of strongly aberrant phenotypes. (F) The thickness of the sensory axon bundle was analyzed by calculating the ratio of bundle thickness in the region between DRGs (light purple in scheme) and bundle thickness in the region where roots enter (dark purple in scheme). This ratio was significantly reduced by around 10% after knockdown of each SynCAM family member compared to GFP-injected control embryos. Schematic drawing depicts the regions where the thickness of axon bundle was measured. One asterisk indicate a p-value<0.05 and two asterisks a p-value<0.01 for the comparison between the values of the GFP-injected control group with the values of experimental groups using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m. (G-J) Spinal cord cross-sections of embryos lacking SynCAM2 (G) or SynCAM3 (H) and of control embryos (I) stained for axonin1. In contrast to control embryos (I), which showed densely packed sensory axon bundles, the bundle of sensory axons after knockdown of SynCAMs was segmented (G, H, arrows). Overview picture shows a spinal cord cross-section stained for axonin1. Box depicts the area of the dorsal root entry zone analyzed in (G-J). (J) Quantification of the number of embryos showing a segmentation of the sensory axon bundle reveals a significant increase after downregulation of SynCAM2 and SynCAM3 compared to controls. Note that knockdown of SynCAM1 did not have an effect on the segmentation of the axon bundle. (E, J) Statistics were done with the two-tailed Fisher exact probability test. One asterisk indicates a p-value<0.05 and two asterisks indicate a p-value<0.01 for the comparison between GFP-injected control groups and experimental groups.

Figure 12

Complex SynCAM cis-/trans-interactions regulate the behavior of axons and growth cones. SynCAMs laterally assemble in cis forming dimers or oligomers of different composition, including homophilic (blue-blue or green-green) or heterophilic (blue-green) combinations. These complexes interact in trans with cis-multimers on neighboring cells. Depending on the composition of the complexes and the underlying trans-interactions, SynCAMs might recruit different intracellular effector molecules and, thus, signal via different intracellular cascades. The intracellular signaling molecules, such as CASK, protein 4.1 or Farp1, interact and modulate the actin cytoskeleton, thereby eliciting specific behavioral responses of axons and growth cones. These might include the switching of axons from one fascicle to another at choice points, a principle known as selective fasciculation. Note that a requirement of the intracellular molecules CASK, protein 4.1 and Farp1 in axon guidance is only hypothetical and needs to be tested.

Figure 13

SynCAMs are required for pathfinding of sensory afferents in the dorsal spinal cord. A) Sensory neurons (blue) located in the DRGs extend their axons (blue) towards the spinal cord where they enter through the dorsal root entry zone (DREZ). In the spinal cord the axons bifurcate to grow along the anterior-posterior axis thereby forming a tight and homogenous fascicle, the sensory axon bundle (grey). (B) After perturbation of SynCAMs in the DRGs in vivo, sensory afferents aberrantly entered the dorsal spinal cord (grey). The sensory axon bundle was of variable thickness, thicker at the sites of roots entering the spinal cord and thinner in the region between DRGs. (C) In cross-sections the sensory axon bundle was segmented due to gaps between axon sub-bundles. The characteristics of this abnormal phenotype (B, C) induced by knockdown of SynCAMs can be explained by a decrease in axon-axon contact and a concomitant impairment in selective fasciculation.

Figures

Figure 1. Purity of SynCAM ectodomains

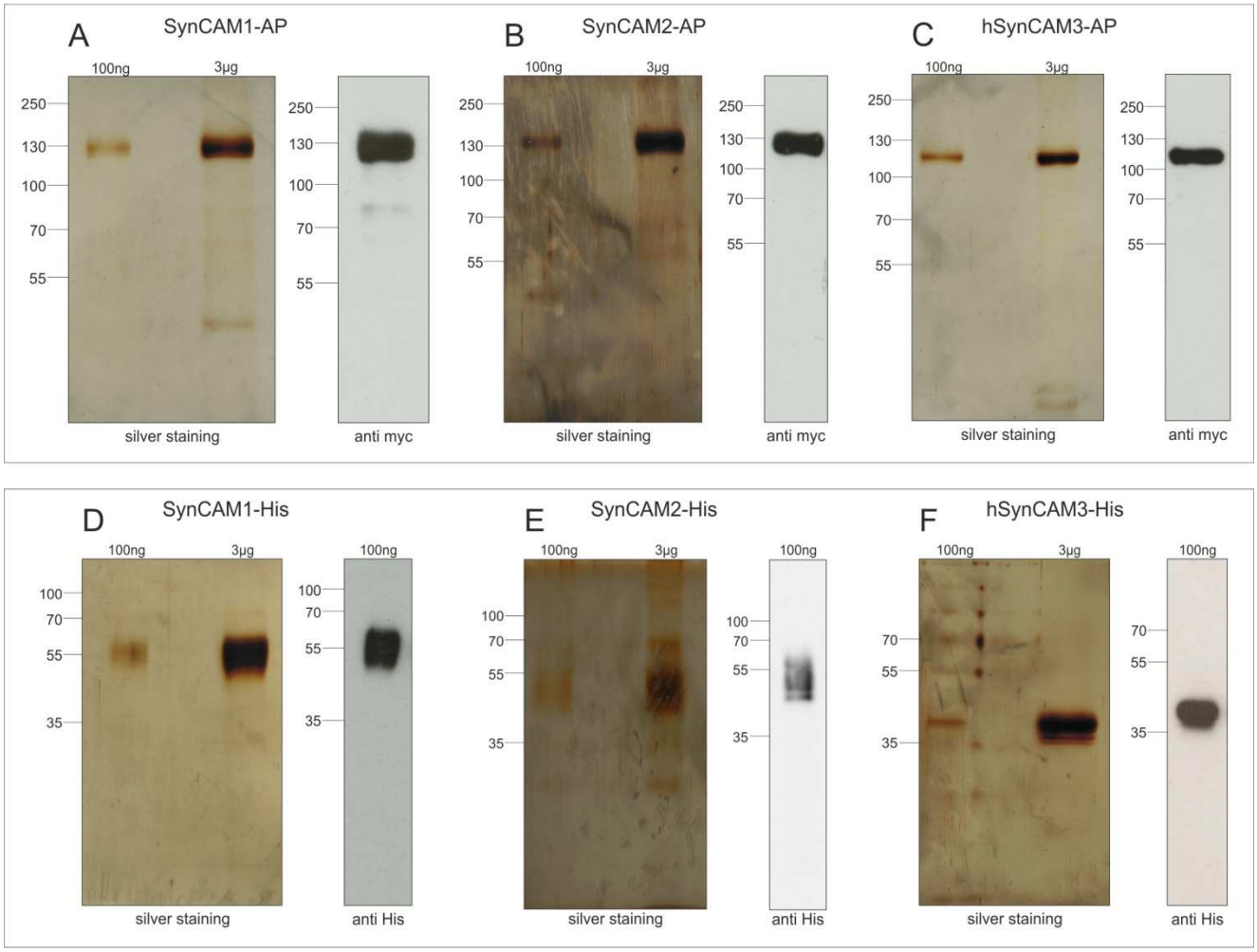


Figure 2. Specificity of SynCAM antibodies

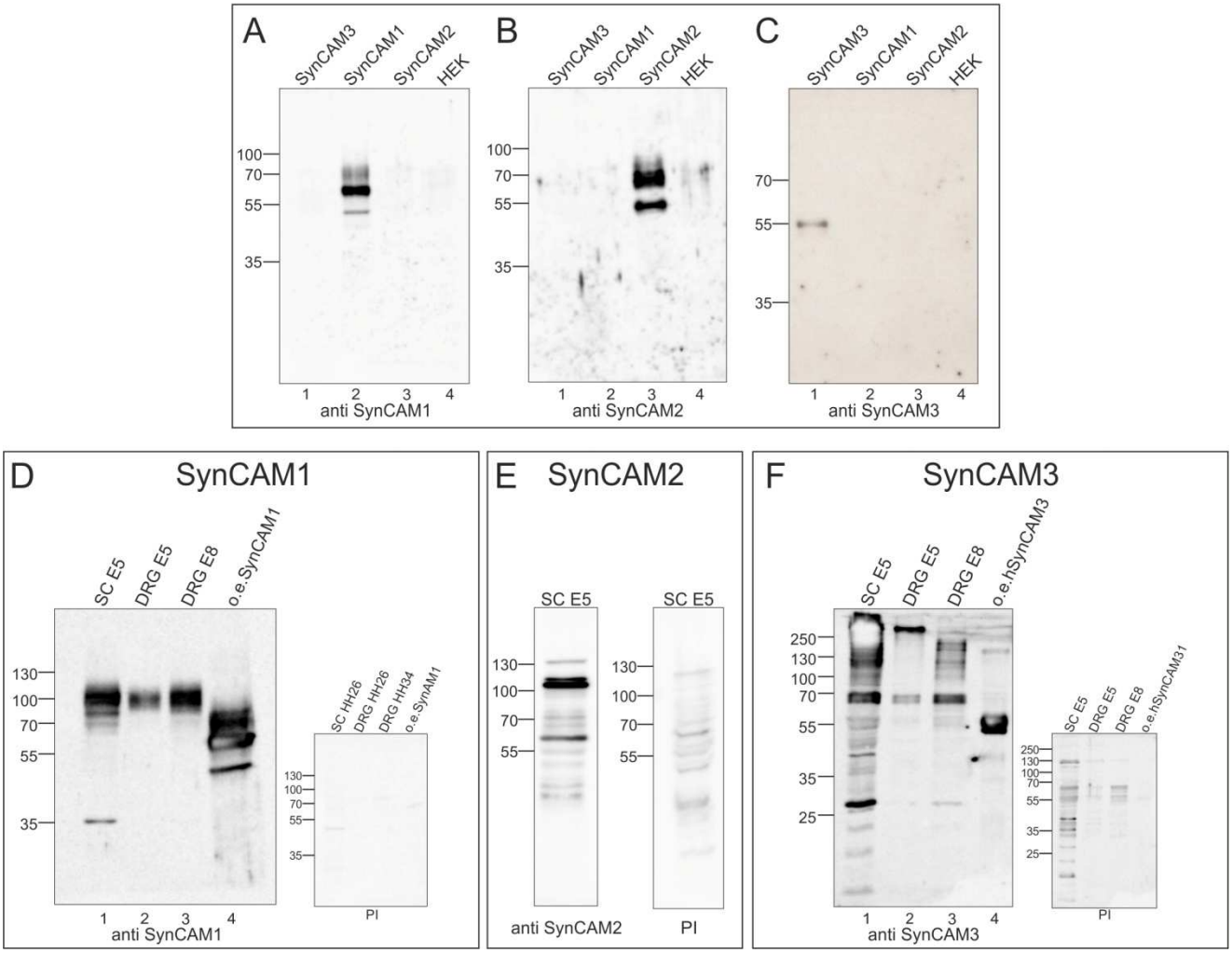


Figure 3. Efficiency and specificity of SynCAM knockdown

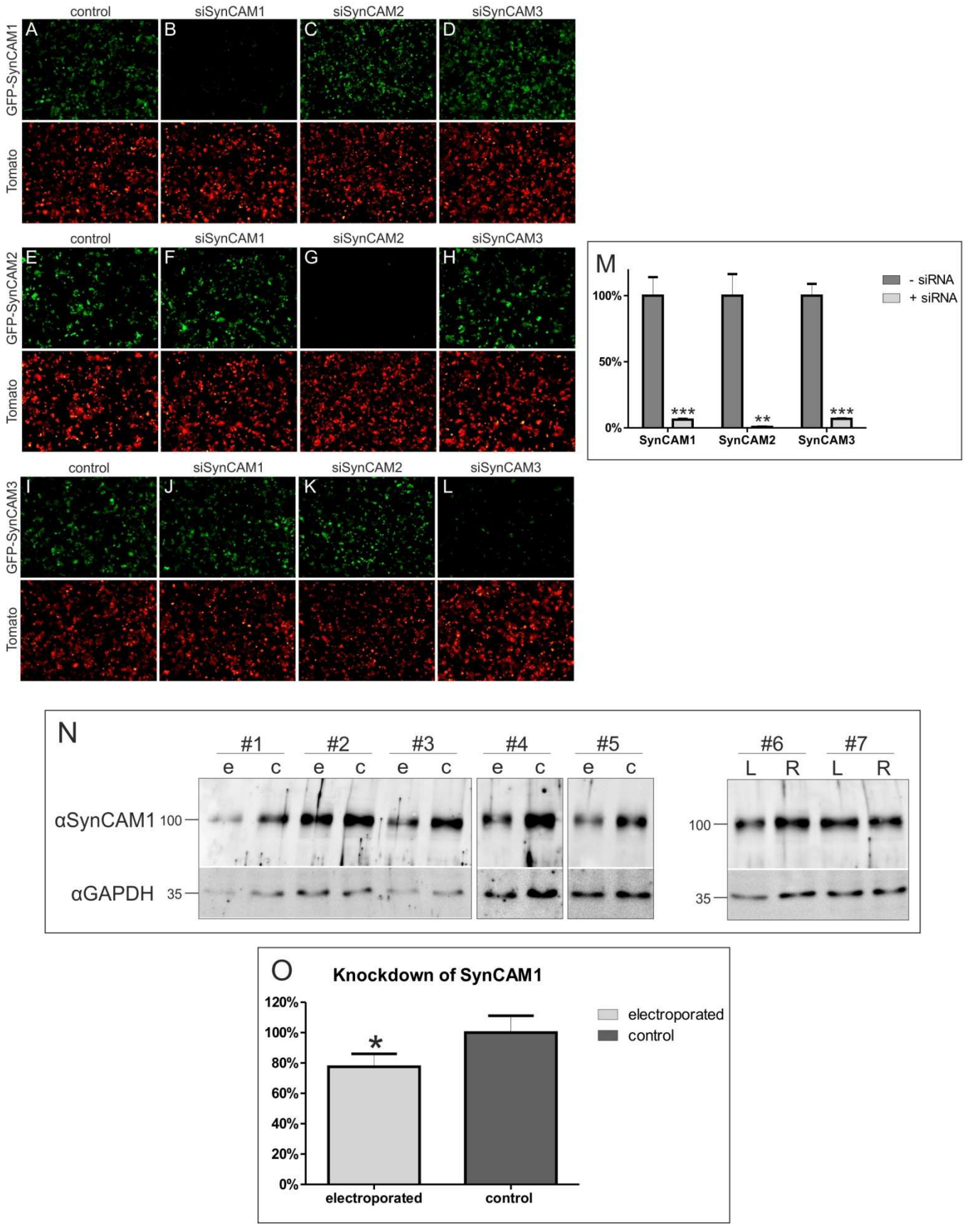


Figure 4. SynCAM1 and SynCAM2 strongly interact in a heterophilic manner

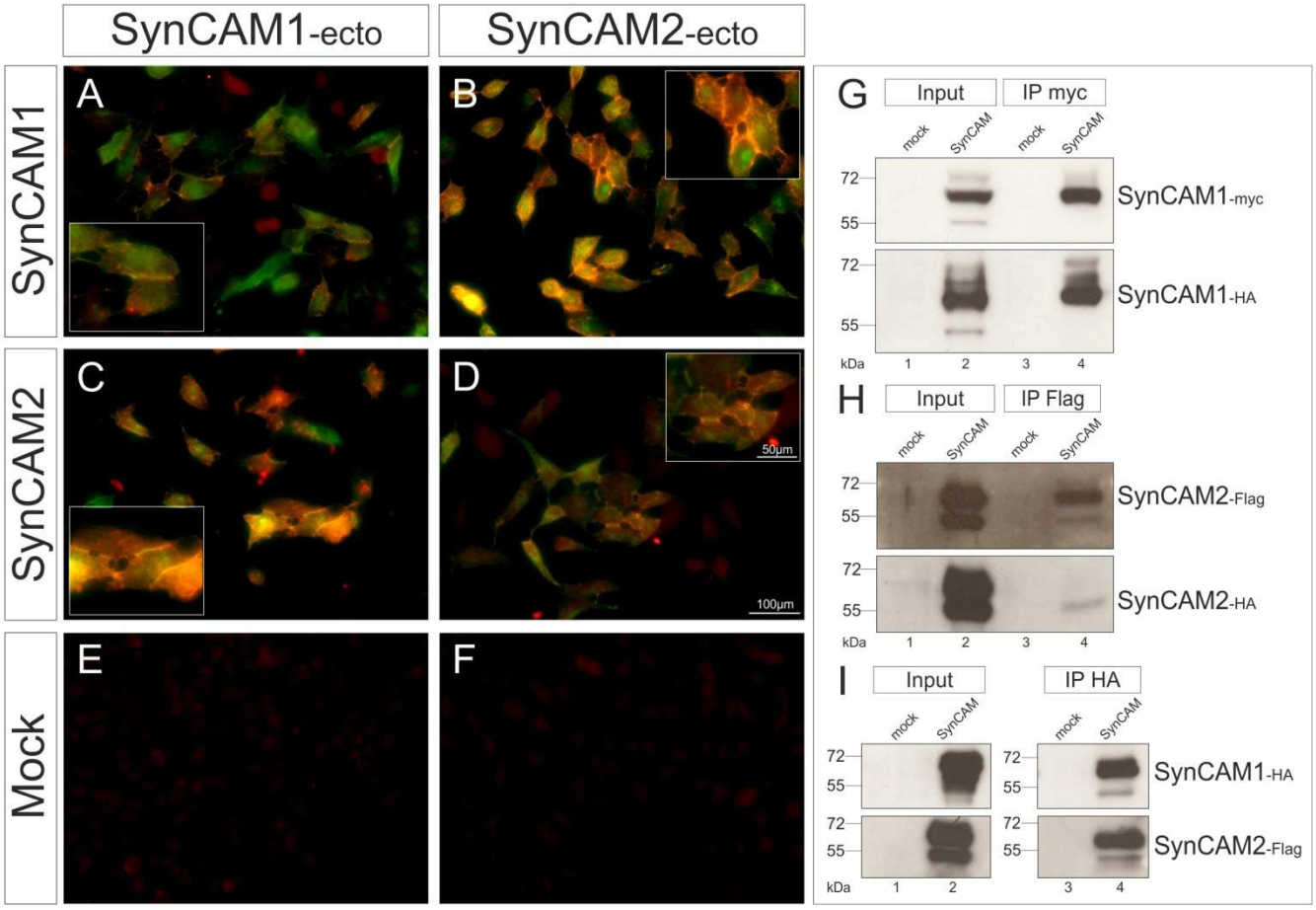


Figure 5. Heterophilic cis-complexes between SynCAM1 and SynCAM2 modify binding in trans

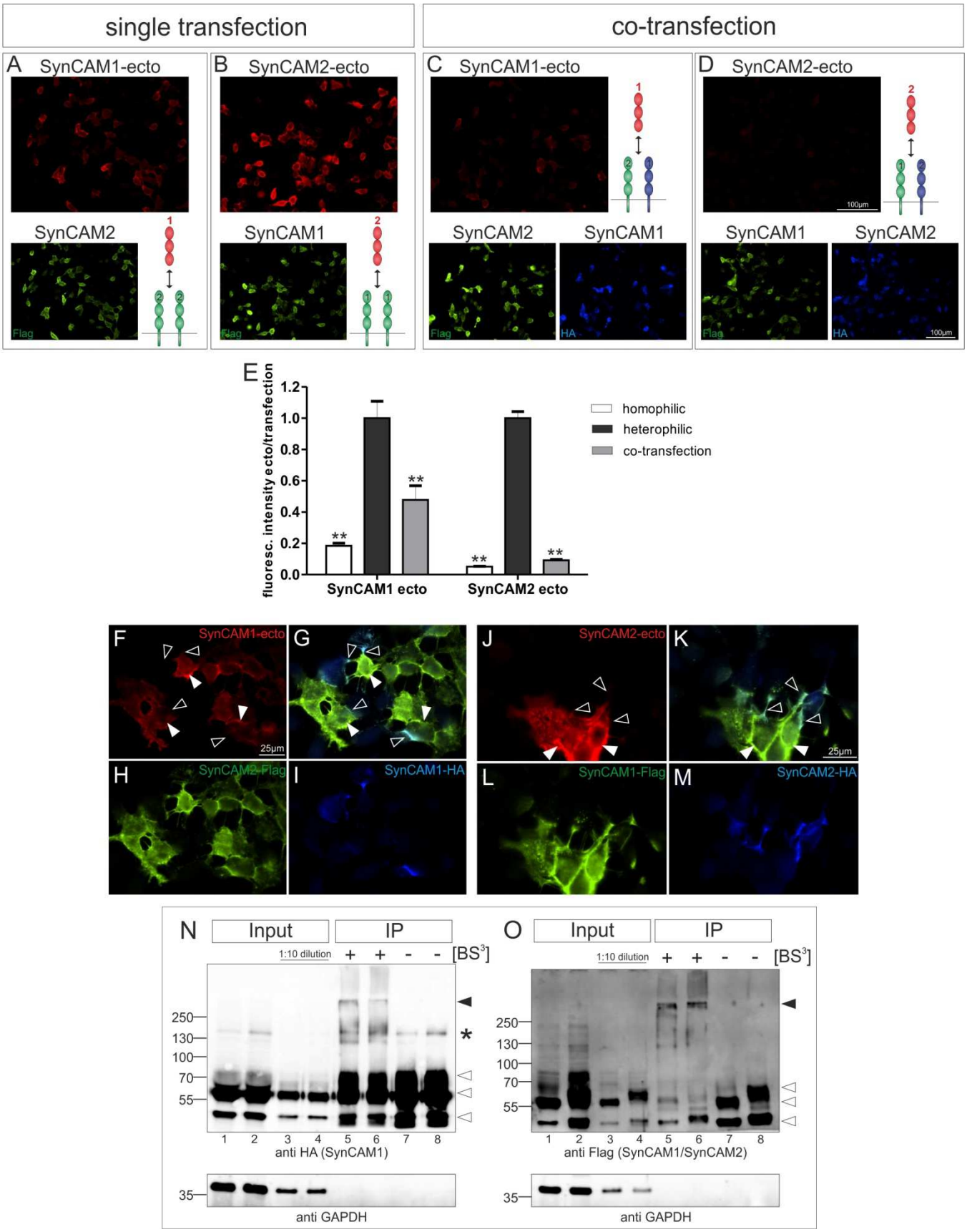


Figure 6. SynCAMs are expressed in DRG sensory neurons, axons and growth cones during the time of sensory axon pathfinding

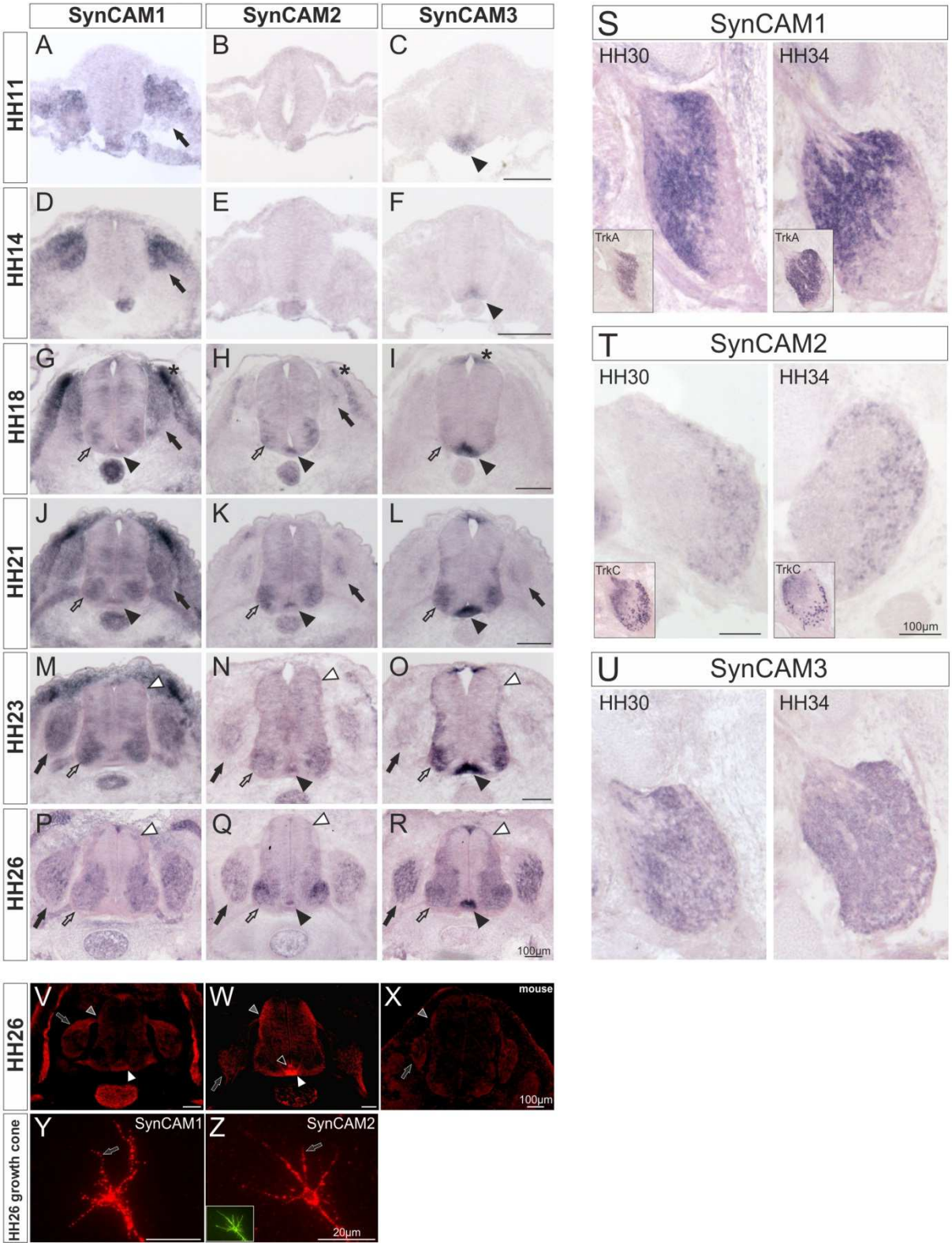
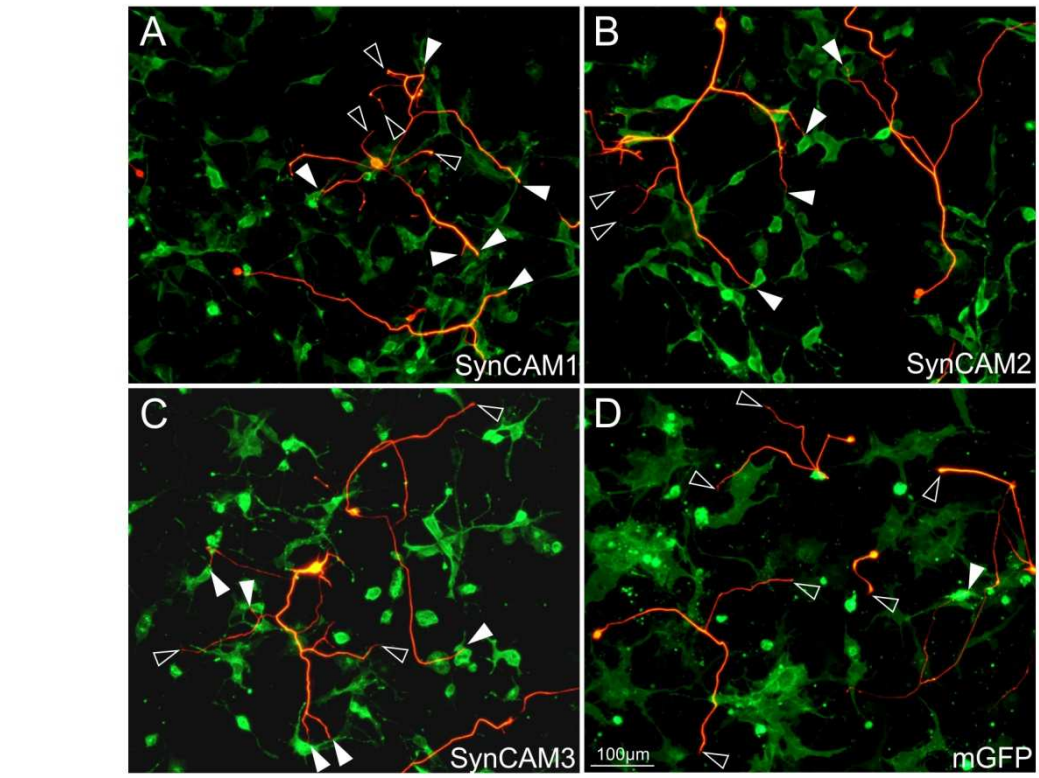


Figure 7. SynCAMs provide an adhesive substrate for sensory axons



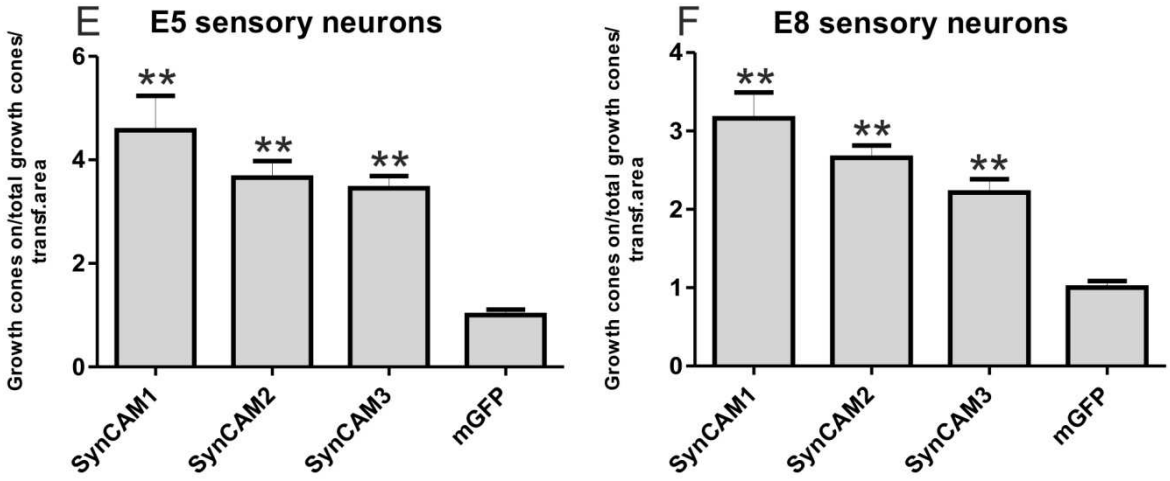


Figure 8. SynCAMs promote neurite outgrowth of E8 but not of E5 sensory neurons

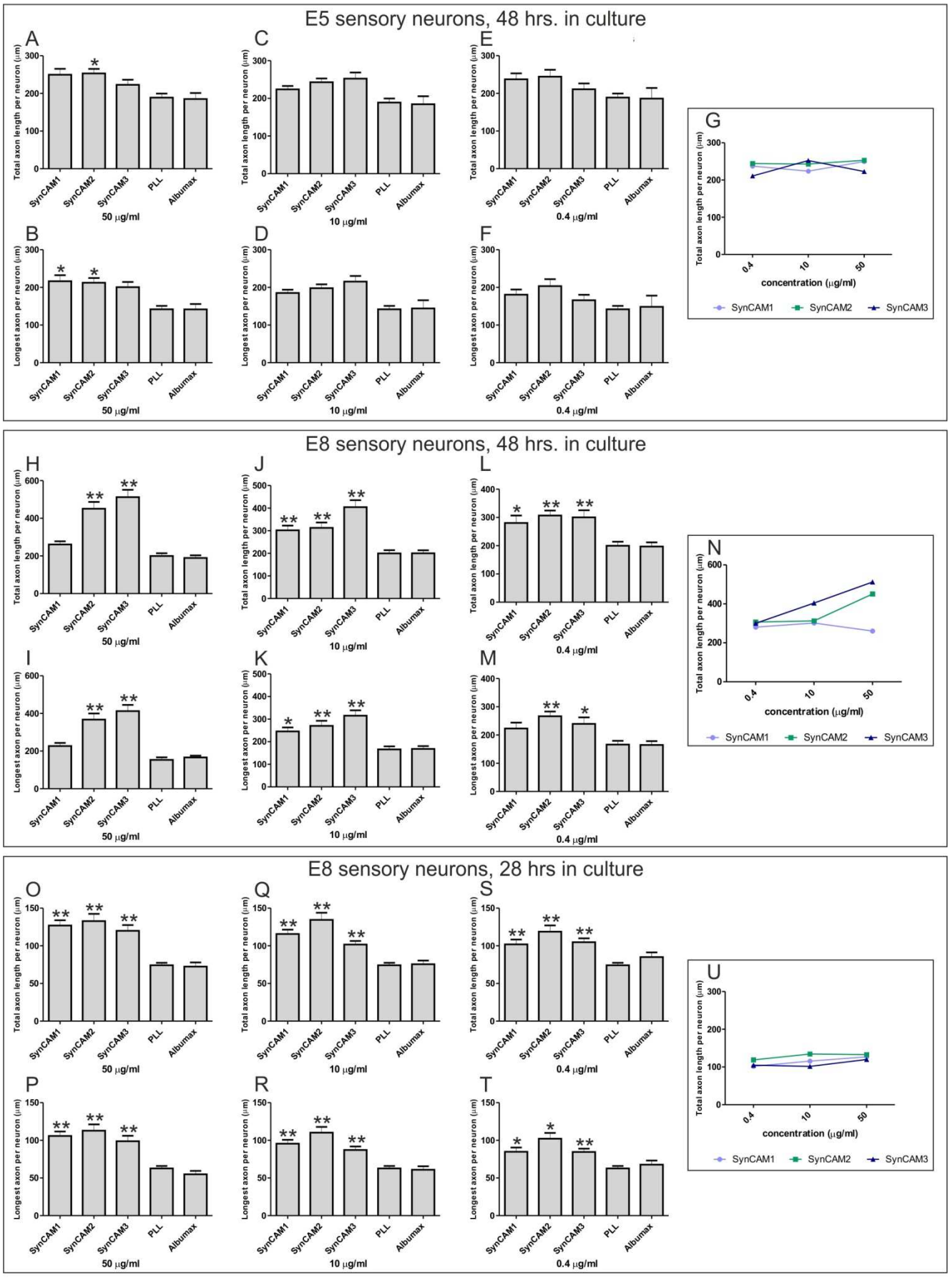


Figure 9. SynCAMs influence axon-axon contacts

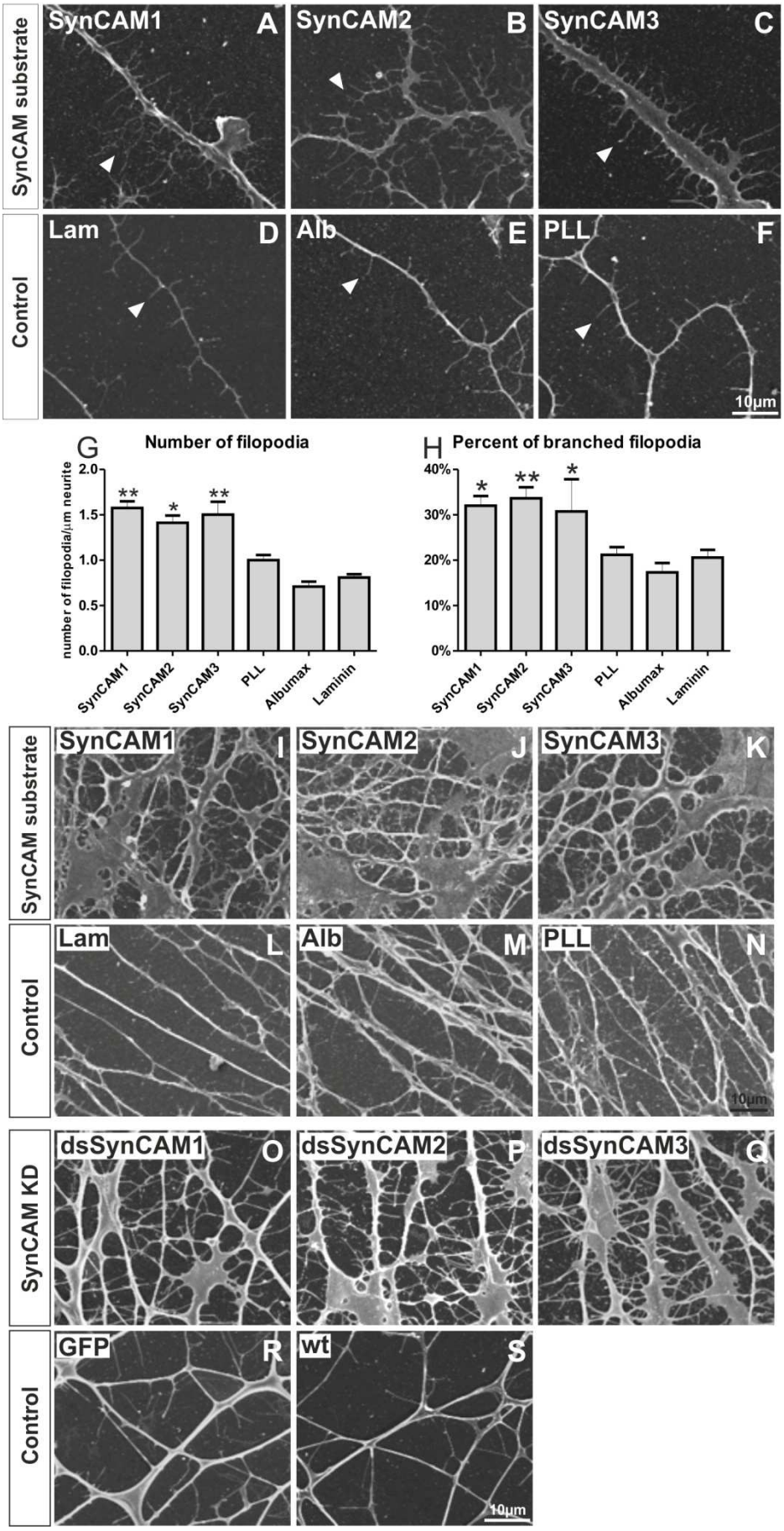


Figure 10. SynCAMs affect growth cone morphology and their distribution on the growth cone surface

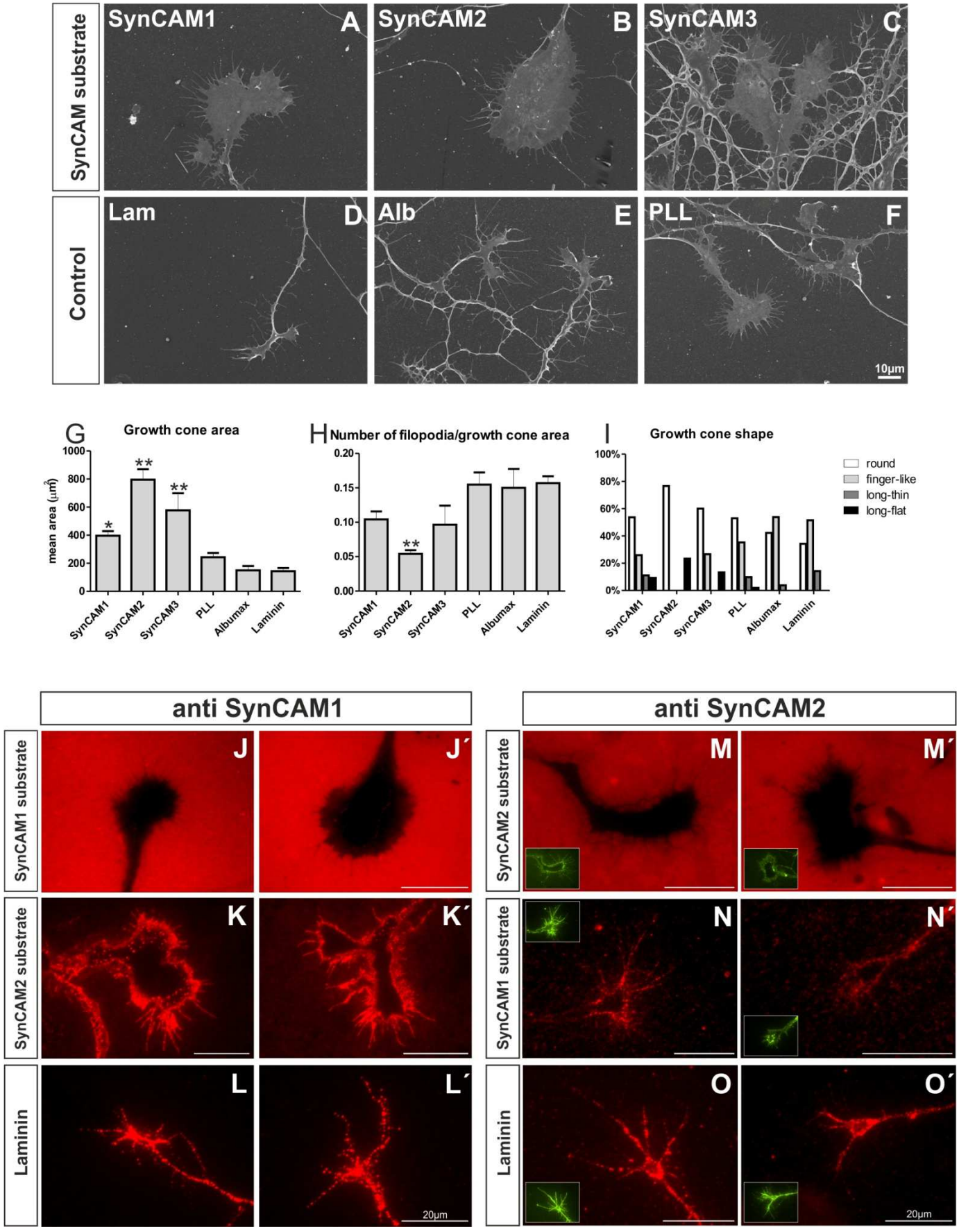


Figure 11. SynCAMs are required for proper pathfinding of sensory afferents

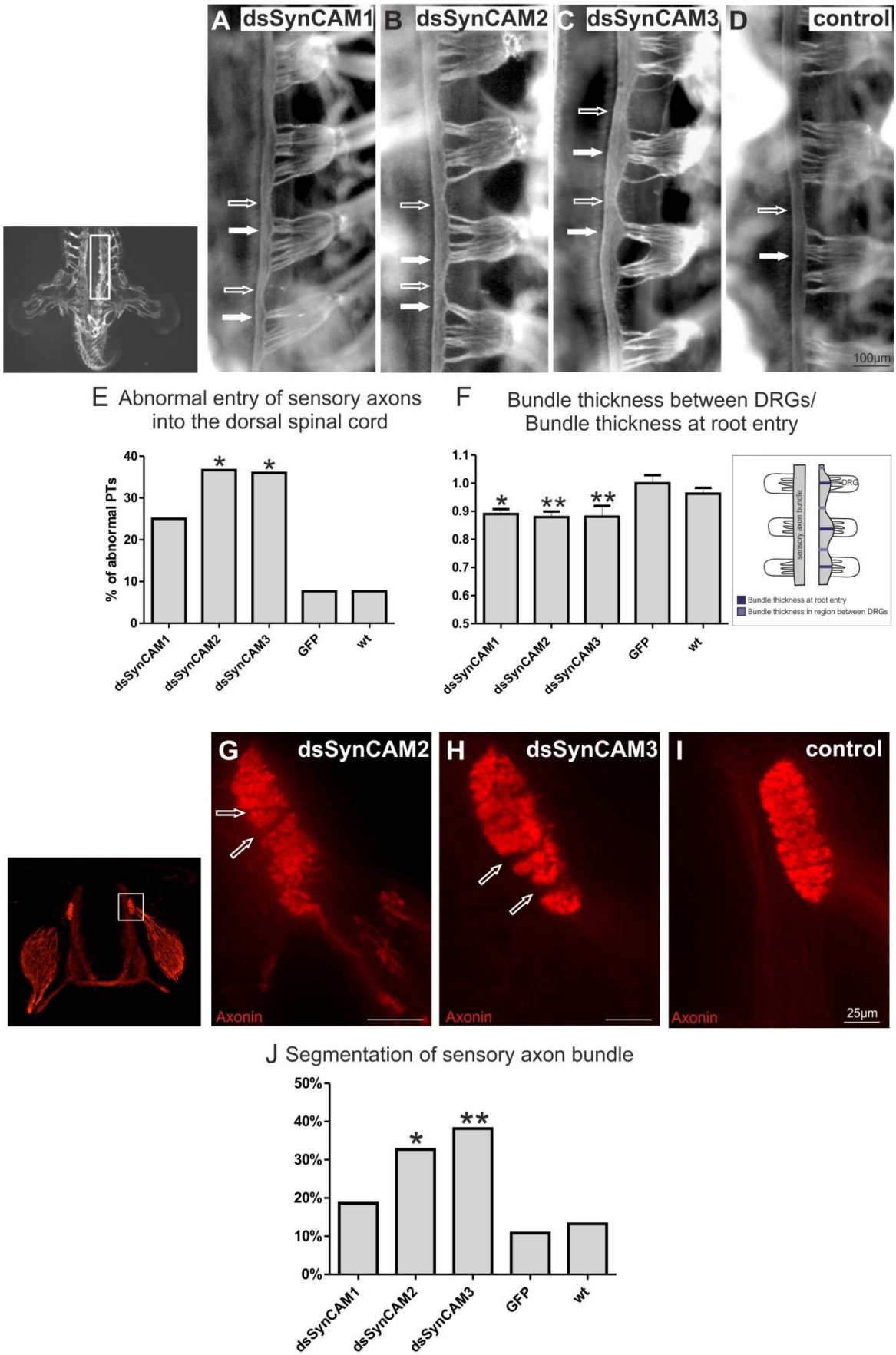


Figure 12. Complex SynCAM cis-/trans-interactions regulate the behavior of axons and growth cones

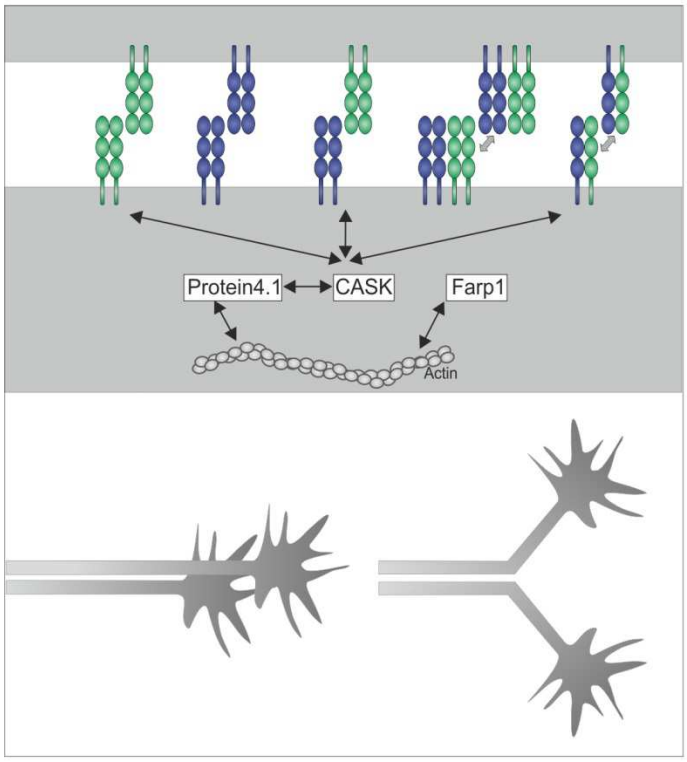
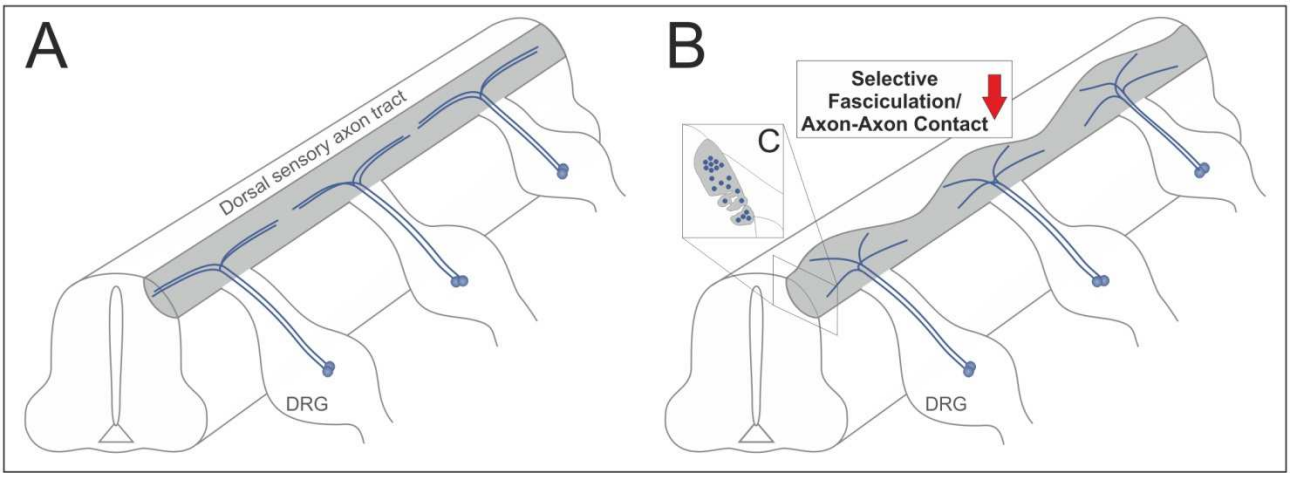


Figure 13. SynCAMs are required for pathfinding of sensory afferents in the dorsal spinal cord



5. Appendix

Additional Methods and Experiments

SynCAMs colocalize at cell-cell contact sites in HeLa cells

To test the different constructs that we used for binding studies and co-immunoprecipitation experiments we transfected HeLa cells using Lipofectamine 2000 (Invitrogen) (see List of plasmids, p. 135). After 24 hours, cells were fixed in 4% formaldehyde and stained with antibodies against the different tags. We used mouse anti-myc (supernatant diluted 1:10; 9E10, Developmental Studies Hybridoma Bank), rabbit anti-HA (1:2000; Rockland) and goat anti-Flag (1:1000; DDDDK, abcam). After both, single and co-transfection, SynCAMs properly localized at the cell surface and cell-cell contact sites, indicating that these molecules at least mediate homophilic adhesion between cells (Fig. 20).

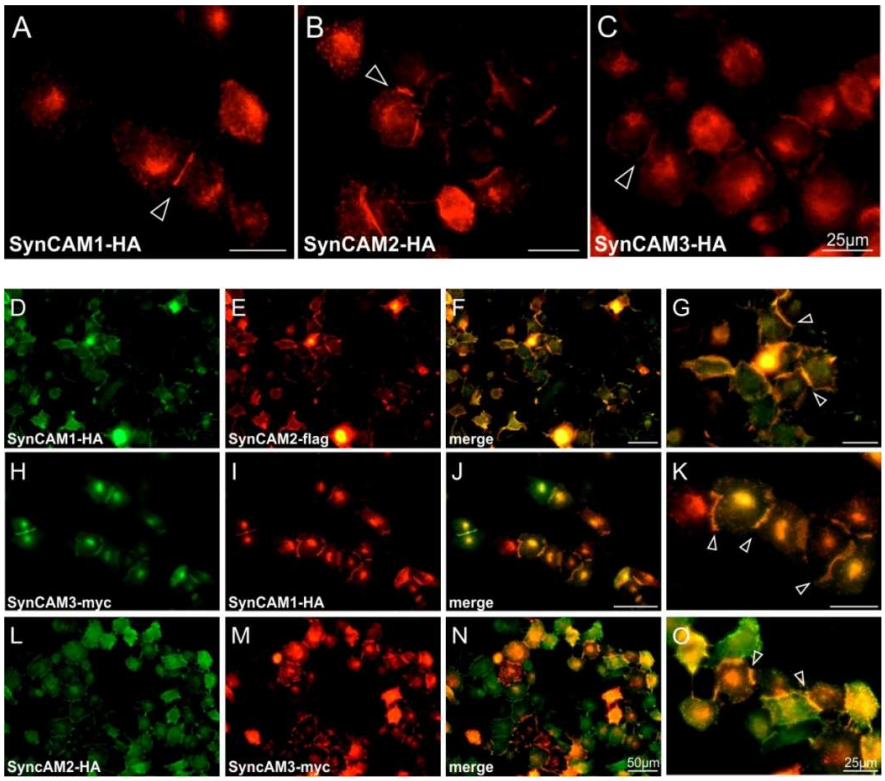


Figure 20. Localization of different SynCAM family members at sites of cell-cell contact. (A-C) Transfection of either SynCAM1, SynCAM2 or SynCAM3 tagged with HA resulted in localization of the proteins at the cell surface with higher levels at cell-cell contact sites (arrowheads). (D-O) After co-transfection of SynCAM1-HA (D, green) and SynCAM2-flag (E, red), SynCAM3-myc (H, green) and SynCAM1-HA (I, red) and SynCAM2-HA (L, green) and SynCAM3-myc (M, red) the proteins colocalized at cell-cell contacts well detectable in the merged pictures (F, J, N). (G, K, O) are higher magnifications of merged pictures (F), (J) and (N), respectively, with arrowheads pointing to colocalization of the different SynCAMs at the plasma membrane involved in cell-cell contact.

To test whether SynCAMs were also able to form heterophilic adhesion complexes we separately transfected HeLa cells with each SynCAM family member and mixed the different cell populations. We found strong accumulation of SynCAMs at cell-cell contacts of mixed cells indicating that SynCAMs recruit each other to assemble into heterophilic adhesion complexes (Fig. 21). Together, these results show that all of our constructs were working as expected and that they localized correctly to the plasma membrane. Furthermore, we could confirm that SynCAMs mediate homo- and heterophilic cell adhesion, as shown previously (Biederer *et al.*, 2002; Fogel *et al.*, 2007).

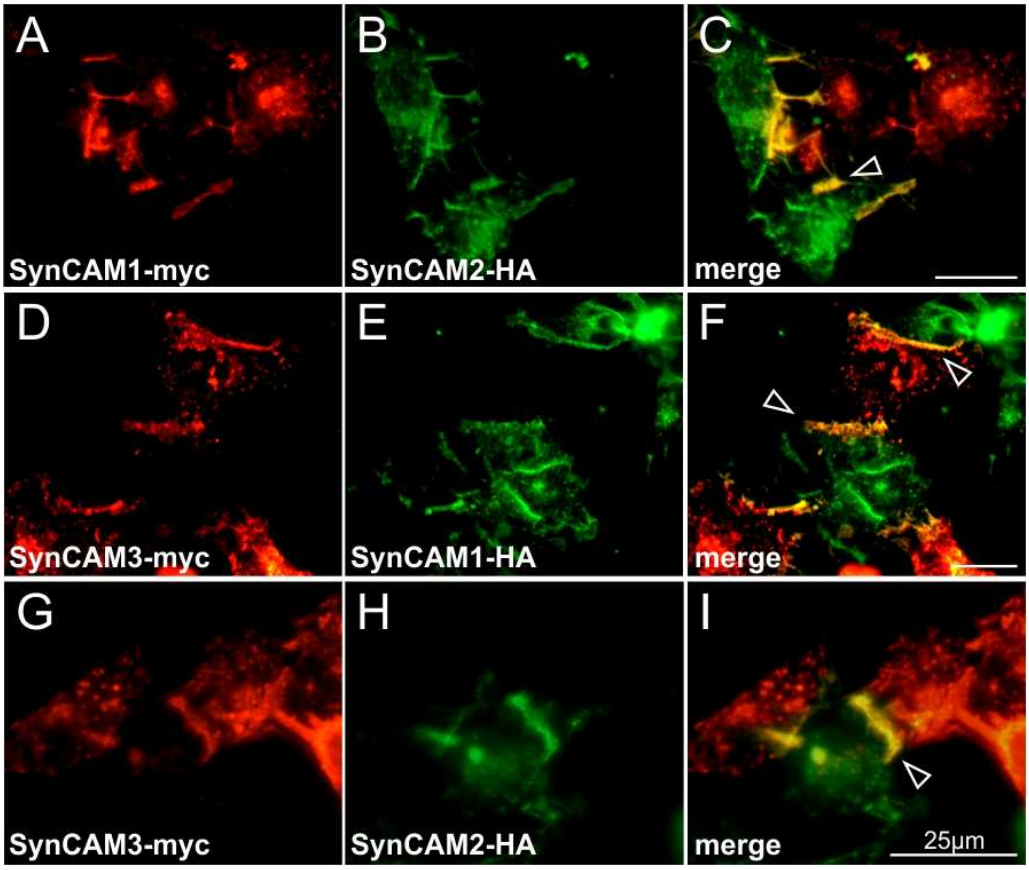


Figure 21. SynCAMs mediate heterophilic cell-cell adhesion. After mixing cell populations that were separately transfected with either SynCAM1-myc (A, red), SynCAM2-HA (B, H, green), SynCAM3-myc (D, G, red) or SynCAM1-HA (E, green), the different SynCAMs colocalized and accumulated at the plasma membrane involved in cell-cell contact which is well detectable in the merged pictures (C, F, I, arrowheads).

Human SynCAM3 interacts in a heterophilic manner in trans but probably only in a homophilic manner in cis

Due to the unavailability of the chicken SynCAM3 sequence we used the human version of this gene for our binding studies. Based on the high degree of sequence similarities between chicken and human SynCAM1 and SynCAM2, respectively, we assumed that chicken and human SynCAM3 would be conserved as well (sequence analysis on NCBI Basic Protein Blast). As for SynCAM1 and SynCAM2, we performed binding studies and co-immunoprecipitation experiments with SynCAM3 (for detailed description of the methods used in these experiments see Material and Methods section in the manuscript). Binding studies with soluble SynCAM3 ectodomains showed no homophilic binding to full-length SynCAM3 but heterophilic binding to full-length SynCAM1- and SynCAM2-transfected HeLa cells (Fig. 22A-D). These results were only partly confirmed by co-immunoprecipitation of co-transfected full-length SynCAMs (for detailed description of the methods used in these experiments see Material and Methods section in the manuscript). SynCAM3 was pulled down with SynCAM1 and SynCAM2, respectively, in line with our own and published results (Fogel *et al.*, 2007; Kakunaga *et al.*, 2005; Niederkofler *et al.*, 2010; Shingai *et al.*, 2003; Thomas *et al.*, 2008) (Fig. 22E, F). Interestingly, SynCAM3 co-immunoprecipitated in a homophilic manner in contrast to the results obtained from the binding study (Fig. 22G). Similar findings were obtained with SynCAM1 (see Fig. 4A and G in Results section in manuscript). These discrepancies could be explained by the fact that during co-immunoprecipitation full-length SynCAMs in lysates are able to from cis-complexes whereas soluble SynCAM ectodomains most probably only bind in trans to full-length SynCAMs expressed on cell membranes. Hence, SynCAM3 and SynCAM1 homophilic interactions might occur mainly in cis and to a lesser extent, if at all, in trans. Furthermore, it is known that homophilic binding between SynCAMs is weaker than heterophilic interactions (Fogel *et al.*, 2007; Niederkofler *et al.*, 2010; Thomas *et al.*, 2008). The binding assay used here might therefore not be sensitive enough for the detection of weak homophilic interactions. This is in line with the results obtained in the binding studies with SynCAM1 and SynCAM2 showing strong heterophilic but only very weak homophilic interactions (see Fig. 4A-D in Results section in manuscript).

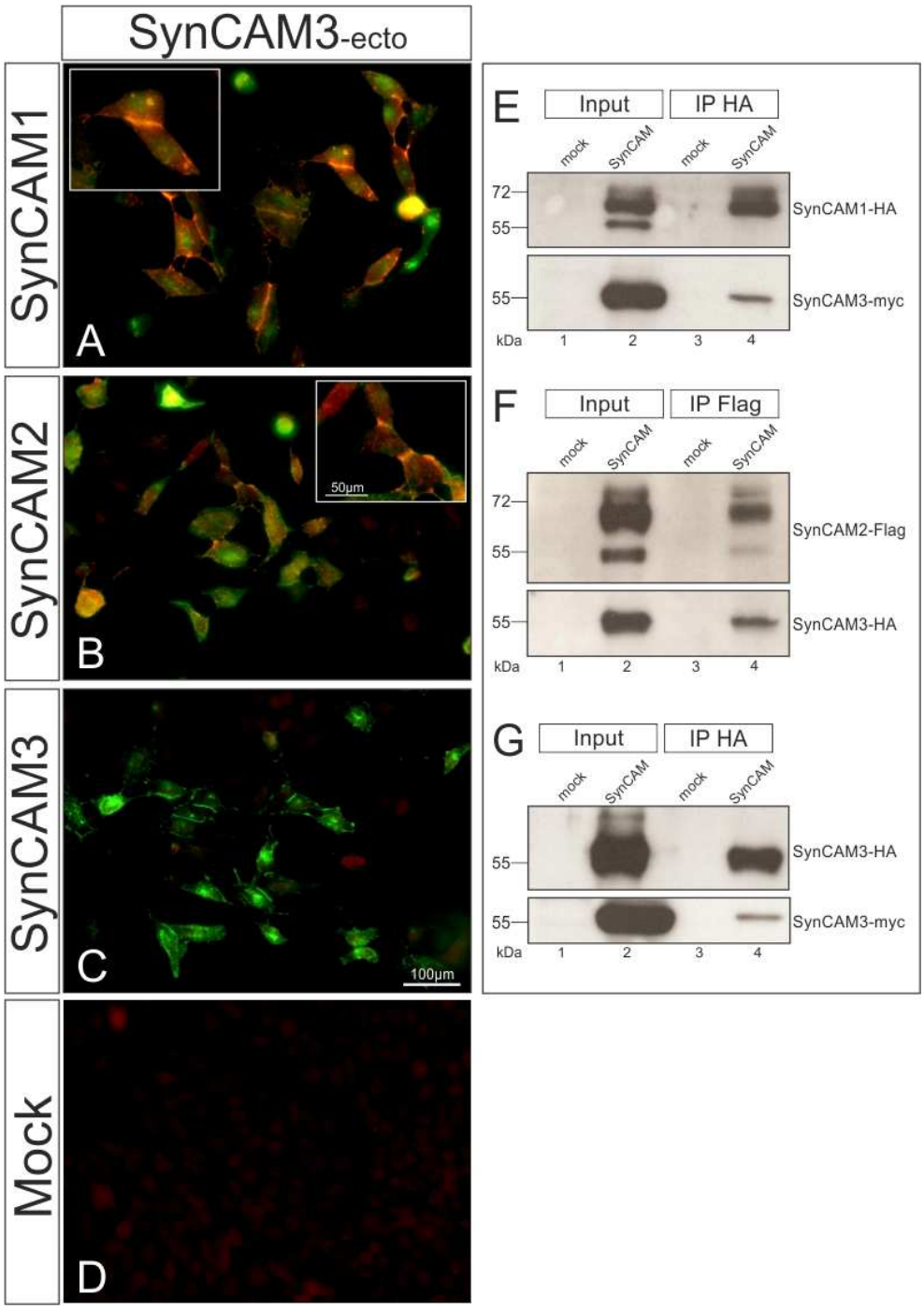


Figure 22. Homo- and heterophilic interactions of SynCAM3. (A-D) Soluble SynCAM3 ectodomains (red) bound in a heterophilic way to full-length SynCAM1-HA- (A, green) and SynCAM2-HA-transfected cells (B, green). No homophilic binding between SynCAM3 ectodomain and SynCAM-3HA-transfected cells (green) could be detected (C). SynCAM3-ectodomain did not bind to mock-transfected cells (D). Insets in (A) and (B) show higher magnifications of cells with bound ectodomains. (E-G) Co-immunoprecipitation of full-length SynCAM3-myc and SynCAM1-HA (E), SynCAM3-HA and SynCAM2-Flag (F) and SynCAM3-myc and SynCAM3-HA (G). Using either anti-HA- (E, G) or anti-Flag-coupled agarose beads (F) SynCAM1 (E), SynCAM2 (F) and SynCAM3 (G) were able to pull down SynCAM3 in a heterophilic and homophilic manner, respectively (lane 4 in E, F and G). Lysates containing mock-transfected HEK293T cells served as negative controls. Anti-myc, anti-HA and anti-Flag antibodies did not stain any unspecific proteins in the mock input lysate (lane 1 in E, F and G). Also, mock transfected cells did not pull down any unspecific proteins (lane 3 in E, F and G). All SynCAMs were successfully coexpressed in HEK293T cells as shown by the input lysate (lane 2 in E, F and G).

Expression of SynCAMs in commissural axons and growth cones supports their involvement in commissural axon guidance

We reported previously that SynCAM1 and SynCAM2 are necessary for proper guidance of post-crossing commissural axons at the midline of the chicken spinal cord (Niederkofler *et al.*, 2010). SynCAM1 and SynCAM2 mRNAs are expressed in commissural neurons (see Fig. 6M, N, P, Q in Results section in manuscript; Niederkofler *et al.*, 2010). Using newly developed antibodies we wanted to compare mRNA and protein expression of SynCAM1 and SynCAM2 on commissural axons and growth cones. Surface staining on dissociated commissural neurons showed the presence of SynCAM1 and SynCAM2 on both, axons and growth cones (Fig. 23).

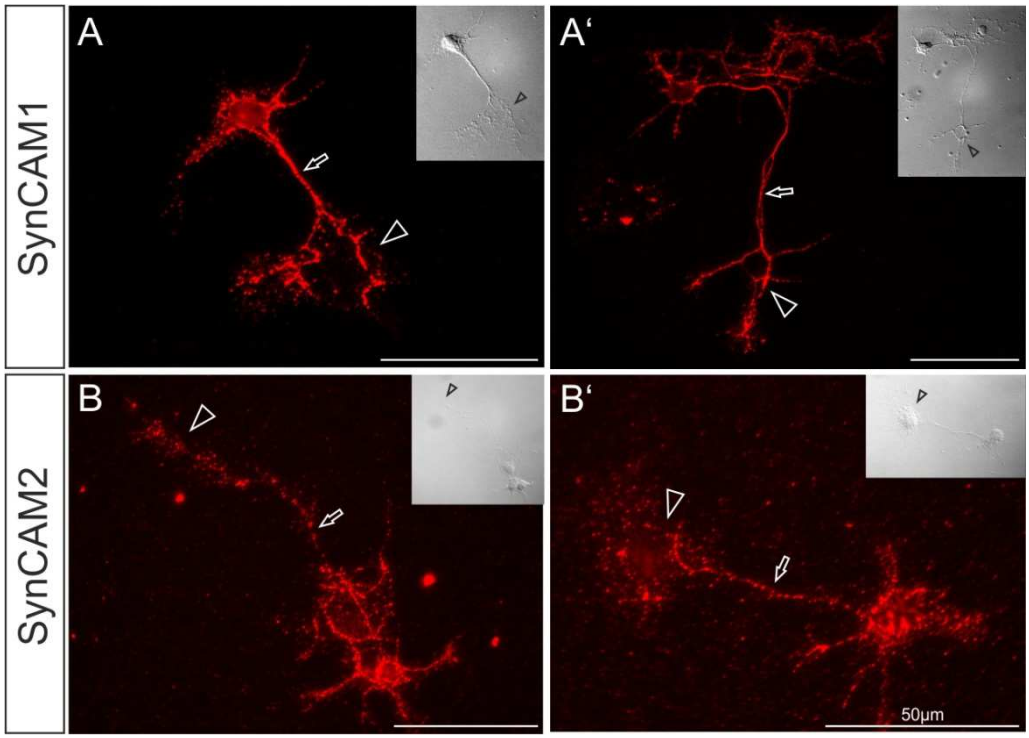


Figure 23. SynCAM1 and SynCAM2 are expressed on the surface of commissural axons and growth cones. Surface staining of SynCAM1 (A, A') and SynCAM2 (B, B') on dissociated commissural neurons revealed the presence of both proteins on commissural axons (arrows) as well as their growth cones (arrowheads). Insets show phase contrast pictures of the commissural neurons stained for SynCAM1 and SynCAM2 with arrowheads pointing to the growth cones.

The fact that commissural axons stalled at the floor-plate exit site after downregulation of axonal SynCAM1 and floor-plate-derived SynCAM2 suggests that the SynCAM1-SynCAM2 trans-interaction plays an important role in commissural axon guidance (Niederkofler *et al.*, 2010). To test whether SynCAM1-SynCAM2 interactions were adhesive in this context, we transfected COS7 cells with different SynCAM family members and co-cultured the cells with dissociated commissural neurons (for detailed description of the methods used in this experiments see Material and Methods section, choice assay, in manuscript). We found a significant increase in growth cones ending on SynCAM1- and SynCAM2-positive COS7 cells compared to cells transfected with MARCKS-GFP (mGFP) (Fig. 24). Hence, as already seen for sensory axons (see Fig. 7 in Results section in manuscript), SynCAMs provide an adhesive substrate for commissural axons.

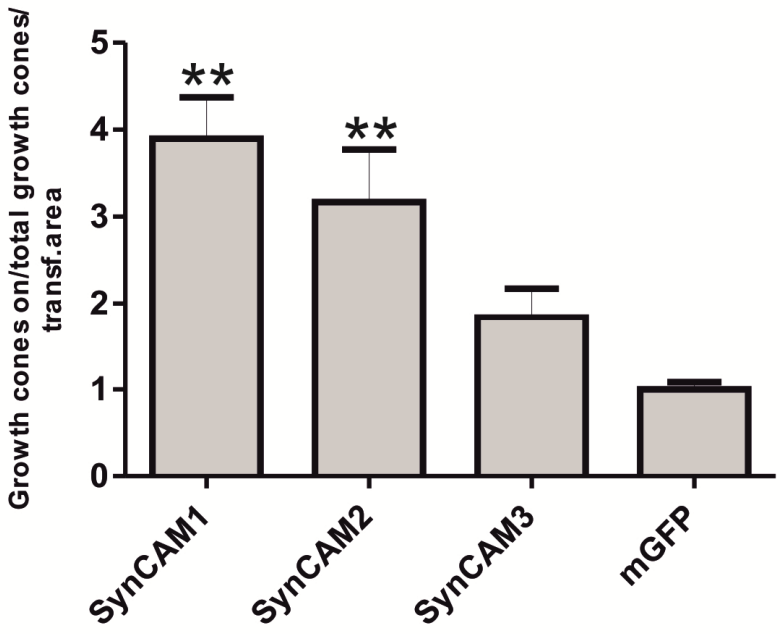


Figure 24. SynCAMs provide an adhesive substrate for commissural axons. Dissociated commissural neurons were cultured on COS7 cells expressing full-length SynCAM1, SynCAM2, SynCAM3 and MARCKS-GFP (mGFP). Calculation of the ratio between growth cones ending on transfected cells and the total number of growth cones revealed that significantly more growth cones stopped on cells expressing SynCAM1 and SynCAM2 compared to cells expressing mGFP. The values for SynCAM3 were not significantly different to the values of mGFP although there was a tendency of axons to preferentially stop on SynCAM3-positive cells. Ratios were normalized to the transfected area. Two asterisks indicate a p-value<0.01 for the comparison between mGFP and all other groups using ANOVA/Tukey HSD post-hoc test. Values are given as mean +/- s.e.m.

Generation of antigens and production of antibodies against the different SynCAMs

For the production of specific antibodies against the different SynCAM family members we used the ectodomains as antigens. In contrast to the cytosolic tail, the ectodomain is not conserved among the different SynCAMs, with maximal identities between 41% and 52% (sequence analysis on NCBI Basic Protein Blast). For SynCAM3 the human sequence was used as the chicken sequence was not available (NCBI and Ensembl.org). SynCAM1 and SynCAM2 show a high degree of sequence conservation between species, about 80% between chicken and human (sequence analysis on NCBI Basic Protein Blast), suggesting that chicken and human SynCAM3 might be similar as well.

To produce antigens we transfected HEK293T cells with chicken SynCAM1, chicken SynCAM2 and human SynCAM3 ectodomains fused to 6xHis-STOP of the pAPtag5 using the calcium-phosphate method. After 24 hours, the medium was changed to serum-free medium (OptiMEM, Gibco). The supernatant containing secreted ectodomains was collected 48 hours later. For SynCAM1 and SynCAM3 a total volume of 300 to 400 mL, and for SynCAM2 a total volume of 2000 mL were collected. The supernatant containing SynCAM2 ectodomains was concentrated 20 times using a tangential filter membrane (Minimate TFF capsule 30 K, Pall Corporation). The antigens were purified by affinity chromatography (FPLC) using Ni-NTA agarose beads (Macherey-Nagel). Prior to loading the samples, 10 mM Imidazole was added. The Ni-NTA column was washed with loading buffer consisting of 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0. Samples were eluted from the Ni-NTA beads with an increasing concentration of imidazole by mixing the loading buffer gradually with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM Imidazole, pH 8.0. SynCAM1 was eluted at a concentration of about 125 mM Imidazole, SynCAM2 at 175 mM Imidazole and SynCAM3 at a concentration of about 75 mM Imidazole. The eluted proteins were collected in multiple fractions. The content of the fractions was visualized on silver-stained gels (Fig. 25). Fractions containing the protein of interest were pooled and dialyzed against PBS (Dialysis Tubing Cellulose Membrane, Sigma).

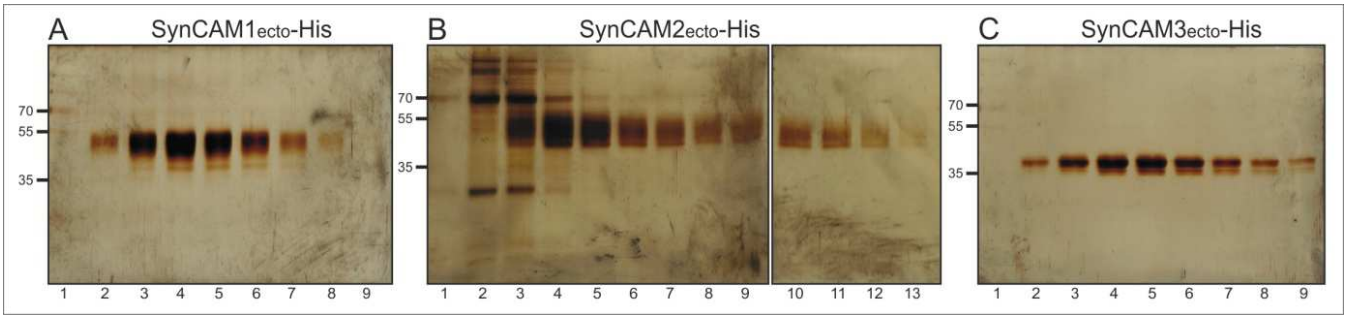


Figure 25. Eluted antigens were collected in multiple fractions. Silver-stained SDS-PAGE gels showing the quantity and purity of the fractions containing SynCAM1 (A), SynCAM2 (B) and SynCAM3 ectodomains (C). Antigens were found at their expected sizes. Only fractions containing the antigen of interest in a high purity were pooled: 3-7 (A), 4-7 and 8-11 (B) and 3-8 (C). Fractions 1-3 in (B) depict contaminations eluted prior to SynCAM2 ectodomain.

The concentration of the pooled fractions was determined using the Bradford method (BioRad Protein Assay, BioRad). The purity of the pooled ectodomains was confirmed on a silver-stained gel and by Western blotting after SDS-PAGE using rabbit anti-His antibody (1:10'000, Rockland) and goat anti-rabbit-HRP antibody (1:10'000; Jackson ImmunoResearch) (see Fig. 1D-F in Material and Methods section in manuscript). In case of SynCAM2 an additional protein at around 70 kDa was detected on silver-stained gels but not on Western blots suggesting that this protein is a contamination (Fig. 26A, B). To identify the contamination the protein was cut out of a Coomassie stained gel and subjected to proteolytic digestion followed by mass spectrometry (performed by the Functional Genomic Center Zurich). The contaminating protein was identified as hemopexin precursor, a serum glycoprotein.

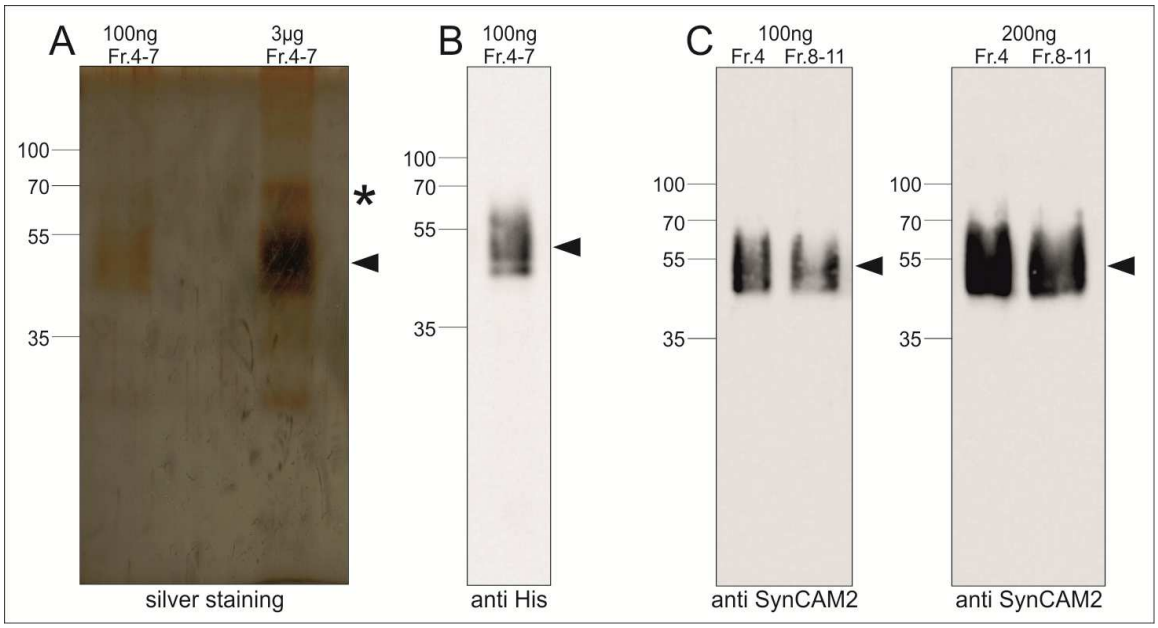


Figure 26. Purity of pooled SynCAM2 antigens and of anti-SynCAM2 antibody serum. (A) An amount of 100 ng and 3 µg of purified SynCAM2 ectodomains (pooled fractions 4-7, see Fig. 25B) were loaded and subjected to SDS-PAGE. Silver staining revealed the purity of the antigen. In addition to SynCAM2 ectodomain at around 55 kDa (arrowheads), a band at 70 kDa (asterisk) could be detected. (B) Western blot loaded with purified SynCAM2 ectodomains (pooled fractions 4-7) and stained for the fused His-tag revealed SynCAM2 at the same height as in (A, arrowhead) but no additional band at 70 kDa. (C) Western blot of purified SynCAM2 ectodomains (pooled fractions 4-7 and 8-11) stained with the anti-SynCAM2 antibody. This antibody recognized SynCAM2 ectodomains at the expected size of 55 kDa (arrowhead) but no additional band at 70 kDa. Even when a higher amount of SynCAM2 ectodomains was loaded (200 ng, right blot, arrowhead) no other bands were detectable. This shows that the anti SynCAM2 serum does not contain contaminating anti-hemopexin precursor antibodies. Pooled fractions 8-11 served as negative control as these fractions did not contain additional contaminating proteins at 70 kDa.

For the production of the antibodies two rabbits per SynCAM were injected each with 50 µg (SynCAM1 and SynCAM3) or 30 µg (SynCAM2) of the purified antigens. In total three booster injections were given at 6 weeks intervals. The specificity of the antibodies was tested on Western blots loaded with HEK293T cell lysates overexpressing the different full-length SynCAM family members. The antibodies did not show crossreactivity among different SynCAM family members (see Fig. 2A-C in Material and Methods section in manuscript). Furthermore, anti SynCAM2 serum did not recognize hemopexin precursor protein, confirming that this serum did not contain antibodies against hemopexin precursor protein (Fig. 26C). The antibody against SynCAM1 recognized endogenous SynCAM1 on Western blot (see Fig. 2D in Material and Methods section in manuscript), dissociated sensory (see Fig. 6Y

in Results section in manuscript) and commissural neurons (Fig. 23A, A') and chicken spinal cord cross sections (Fig. 6V in Results section in manuscript). So far, SynCAM2 antibody stained endogenous SynCAM2 on dissociated sensory (see Fig. 6Z in Results section in manuscript) and commissural neurons (Fig. 23B, B') and on spinal cord cross sections (Fig. 6W in Results section in manuscript). On Western blot multiple bands were detected (see Fig 2E in Material and Methods section in manuscript). SynCAM3 was recognized on mouse but not on chicken spinal cord sections using the antibody against human SynCAM3 (see Fig. 6X in Results section in manuscript and data not shown). This antibody did not clearly recognize endogenous chicken SynCAM3 on Western blot (see Fig. 2F in Material and Methods section in manuscript), suggesting that SynCAM3 might not be as highly conserved between species as SynCAM1 and SynCAM2.

List of plasmids

Constructs used for production of ectodomains, binding studies, co-immunoprecipitation and choice assay

Insert	Vector	Experiment
SynCAM1ecto-6xhis-STOP	APtag-5	antigen production/surface coating: ectodomains
SynCAM2ecto-6xhis-STOP	APtag-5	antigen production/surface coating: ectodomains
humanSynCAM2ecto-6xhis-STOP	APtag-5	antigen production/surface coating: ectodomains
SynCAM1ecto-AP-myc-6xhis	APtag-5	binding studies: ectodomains
SynCAM2ecto-AP-myc-6xhis	APtag-5	binding studies: ectodomains
humanSynCAM2ecto-AP-myc-6xhis	APtag-5	binding studies: ectodomains
f.l.SynCAM1-HA-STOP	pcDNA3.1(-)myc-hisA	binding studies co-binding studies co-immunoprecipitation choice assay
f.l.SynCAM2-HA-STOP	pCAGGs	binding studies co-binding studies co-immunoprecipitation choice assay
human f.l.SynCAM3-HA-STOP	pcDNA3.1(-)myc-hisA	binding studies co-immunoprecipitation choice assay
f.l.SynCAM1-Flag-STOP	pcDNA3.1(-)myc-hisA	co-binding studies
f.l.SynCAM2-Flag-STOP	pCAGGs	co-binding studies co-immunoprecipitation
f.l.SynCAM1-myc-6xhis	pcDNA3.1(-)myc-hisA	co-immunoprecipitation
Human f.l.SynCAM3-myc-6xhis	pcDNA3.1(-)myc-hisA	co-immunoprecipitation

Chicken Expressed Sequence Tags (ChESTs) and cloned gene fragments

Insert	ChEST number	Vector	Experiment
SynCAM1 3'UTR	ChEST583g11	pBluescript II KS+	ISH and dsRNA preparation
SynCAM2 CDS incl. STOP/3'UTR	ChEST114o11	pBluescript II KS+	ISH
SynCAM2 3'UTR	ChEST96i3	pBluescript II KS+	dsRNA preparation
SynCAM3 3'UTR	ChEST478g10	pBluescript II KS+	ISH and dsRNA preparation

ISH= in situ hybridization

6. References (for 1. Introduction, Sensory neural circuit formation and 5. Appendix)

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Publications

● Jeannine Frei and Esther T. Stoeckli (submitted). *SynCAMs extend their functions beyond the synapse.*

● Jeannine Frei and Esther T. Stoeckli (in preparation). *The Synaptic Cell Adhesion Molecules SynCAMs Are Involved in Early Sensory Axon Pathfinding.*

● Jeannine Frei and Esther T. Stoeckli (in preparation). *Synaptic Cell Adhesion Molecules – A Link between Neural Circuit Formation and Neurodevelopmental Diseases.*

Abstracts

● *Role of Nectin-like Molecules/SynCAMs in Neural Circuit Formation*, ZNZ Symposium 2011, Zürich.

● *Nectin-like Molecules/SynCAMs Contribute to Sensory Neural Circuit Formation*, SSN Meeting 2012, Zürich.

● *SynCAMs/Nectin-like Molecules Are Required for Early Stages of Neural Circuit Formation,* FENS meeting 2012, Barcelona.

● *SynCAMs/Nectin-like Molecules are Required for Early Stages of Neural Circuit Formation,* SSN Meeting 2013, Genf.

● *The Synaptic Cell Adhesion Molecules SynCAMs Are Involved in Early Sensory Axon Pathfinding,* SfN Neuroscience Meeting 2013, San Diego.